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METAL RESISTANCE IN ALCALIGENES MARINUS BY PRODUCTION OF EXTRACELLULAR BINDING AGENTS

JAMES BEAVEN RAKE

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METAL RESISTANCE IN *Alcaligenes marinus* BY PRODUCTION OF EXTRACELLULAR BINDING AGENTS

BY

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This dissertation is dedicated to Geoffrey and Helen who made science a passion for me, and most of all to Barbara whose love and perseverance made it possible.

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ABSTRACT

METAL RESISTANCE IN Alcaligenes marinus BY
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A Ni-resistant marine bacterium which formed enlarged, vacuolated cells (megalomorphs) under Ni-stress previously was classified as Achromobacter sp., Arthrobacter marinus, and Pseudomonas marina. Arthrobacter marinus (ATCC 25374) and independently isolated Pseudomonas marina (ATCC 27129) were related at the species level by numerical taxonomy of 130 characteristics. They were gram-negative, non-pigmented, non-proteolytic, aerobic, oxidative, and metabolically versatile. The G+C content of cell DNA was 63.0 $\pm$ 0.5 moles percent. The bacterium was peritrichously, not polarly flagellated, and the cycle of morphology and gram stain response of Arthrobacter was absent. Of existing genera, Alcaligenes was the best placement. Alcaligenes marinus may be the prototype of a common marine bacterium.

In a complex broth medium (0.5 g peptone, 0.5 g yeast extract per liter 75 % artificial seawater) A. marinus was uneffected by Ni to a concentration (1 X 10^{-4}M) above which growth rate and yield were progressively depressed to 6 X 10^{-4}M, above which no growth occurred. Cells inhibited by Ni remained viable. At partially inhibitory Ni concentrations, growth

commenced rapidly without an appreciable increase of lag time. In higher inhibitory Ni concentrations cell growth without division gave rise to megalomorphs.

By contrast, in a glucose - mineral salts - seawater broth medium the minimum inhibitory dose was reduced to 1 X 10^{-5}M. Above that Ni concentration growth occurred with nearly normal kinetics, but after a lag period proportional to the Ni concentration. Megalomorphic cells were not observed in this medium at any Ni concentration.

The formation of megalomorphs in complex medium was proposed to be due to bypassing of a Ni-blocked biosynthetic pathway by preformed metabolic intermediates provided by the complex medium. Megalomorphs did not form in the glucose medium as it lacked preformed nutrients.

The Ni-induced lag phase in glucose medium was due to inhibition of both growth and division without loss of cell viability or metabolic activity. No evidence for adaptation (genetic or somatic) to Ni during lag was found. Lag time was inversely proportional to inoculum size. Ni-detoxification during lag was demonstrable by a time proportional reduction of Ni toxicity to a second inoculum by a first inoculum removed by filtration. Detoxification was not due to precipitation of the metal or uptake by cells. Uptake of Ni by A. marinus was low but quantifiable, slow, non-energy dependent, decreased at high Ni concentrations, and indicated an energy-dependent Ni exclusion system. The evidence supported a mechanism of lag phase termination in which A. marinus produced a Ni-complexing agent which detoxified the Ni. When the concentration of

uncomplexed Ni fell below the inhibitory threshold, cell division was derepressed and growth occurred with normal kinetics.

Complexation of Ni during A. marinus lag in glucose medium was demonstrable by differential pulse polarography. During the lag the electrochemical peak of free Ni ($E_{1/2}$=-1.12 v) was both decreased in height indicating less free ion and shifted to a more negative potential ($\Delta E_{1/2}$ up to -0.025 v) indicative of complexation.

Isolation of the organometallic complexes formed by A. marinus was attempted by membrane ultrafiltration, gel filtration, and chelating resin chromatography. As complexes were not detected by these techniques the complexes were of less than 500 dalton molecular weight or of sufficiently low stability to dissociate during isolation. Calculation of stability constants from polarographic data gave pK_m values from 3.3 to 9.3 (dependent on the unknown ligand number of the complex), indicating dissociation due to low stability, such that exchange reactions could occur with chromatography and ultrafiltration media.

CHAPTER I

INTRODUCTION

The potential impact of naturally-occurring or man-induced hazardous agents in the oceans and the limitation of the present understanding of them has become an impetus for the study of the input, persistence, cycling, and food chain bioamplification of toxic, teratogenic, and mutagenic elements and compounds. Some environmental hazards are associated with human activities such as: fission by-product radionuclides, polychlorobiphenyls, petroleum spills, and lead from internal combustion fuels (Goldberg, 1975). There are also natural sources of hazardous materials some of which may equal or exceed anthropogenic inputs such as natural petroleum seeps and heavy metals derived from natural weathering processes (Goldberg, 1975). Mercury from human industrial sources has been identified as the source of the methyl mercury intoxication epidemic in the Minamata Bay district of Japan (Goldberg, 1975). But the occurrence of statutorially unacceptable concentrations of Hg in swordfish on the basis of retrospective studies of museum specimens has been attributed to non-human sources (Miller et al., 1972).

In order to understand the cycling of metal elements in the oceans, knowledge of metal behavior from a broad range of scientific disciplines is necessary, such as: the geological study of the occurrence of metal-bearing deposits and their weathering (Nichols, 1976); the analytic and solution chemical

study of the river and ocean transport, precipitation, and remobilization of metals, and their aqueous phase speciation (Turekian, 1977); and the biologic study of the uptake, excretion, accumulation, transformation, and transport of metals (Bowen, 1966). These and other inputs are required before understanding of the occurrence, role, and possible hazards of any toxic element can be derived.

The biological effect of toxic and nutrient trace metals is modulated by the speciation of the metal in solution, particularly by organic complexing agents (Johnston, 1964). Organic complexation has been implicated in both the increased availability of iron to phytoplankton (Barber and Ryther, 1969), and in the reduction of copper toxicity to phytoplankton (Jackson and Morgan, 1978).

The present study was undertaken to further elucidate the mode of interaction of a marine bacterium with a heavy metal, Ni. This system was investigated previously for morphological and physiological effects of the metal (Cobet, 1968), and the cell wall as the site of metal action (Gonye, 1972). A determination of how a marine bacterium responded to a toxic metal was undertaken, whether by modification of the cell or by modification of the cell surroundings by the production of metal complexing agents.

CHAPTER II

LITERATURE REVIEW

Trace Metal Geochemistry

Analysis of the ionic constituents of seawater shows that 99.9 % of the solids in seawater can be accounted for by 11 ionic species, five cations (Na^+, K^+, Mg^{++}, Ca^{++}, and Sr^{++}) and six anions (Cl^-, $SO_4^=$, HCO_3^-, Br^-, $BO_3^=$, and F^-) (Brewer, 1975). The major constituents have the property that they are remarkably constant in relative ratios to Cl when analyzed in water samples from widely separated areas of the oceans, even when the total salinity in the samples varies. On this basis, these major components have been termed "conservative elements". The remaining 0.1 % of dissolved solids is comprised of greater or lesser amounts of virtually every stable isotope of the periodic chart as well as unstable isotopes which are part of natural decay series (Brewer, 1975). More than 66 of the 94 naturally occurring elements have been measured in seawater. The remainder are doubtless present in undetectable amounts (Goldberg, 1975). In marked contrast to the conservative major elements, the minor elements vary widely in concentration when sampled over transects in space or time (Brewer, 1975). This difference in conservation of composition is related to differences in the residence times of the element in the marine system. Residence time is the average period of time which a given atom of the element remains in solution between its introduction as by stream flow and its loss from

the system as by precipitation (Stumm and Morgan, 1970; Goldberg, 1963; Brewer and Yeats, 1977). Residence times for major elements have values which compare with geological times: Na 2.6×10^8 yr, Sr 1.9×10^7 yr (Stumm and Morgan, 1970). By contrast the non-conservative elements have short residence times: Ni 9×10^4 yr, Cu 2×10^4 yr, Hg 8×10^4 yr, Pb 4×10^2 yr (Brewer, 1976). Another measure of the high reactivity of the non-conservative elements is the relative undersaturation of a given species in seawater compared to its potential saturation concentration (Goldberg, 1963). Na is about 10-fold undersaturated and Ca and Sr are at or near saturation in seawater. By contrast, Ni is undersaturated by a factor of 10^4 to 10^5. The rapid turnover and extreme undersaturation of the minor elements is due to their high reactivity. Both inorganic and biological reactions lead to the removal of the element from solution and ultimately to its deposition into sediments.

In order to understand the processes of marine and freshwater transport and the fate of metals, it is necessary to define the speciation of the metal in solution: free ions, inorganic complexes, organic complexes, or colloids. Further, the prediction of the behavior of metals at interfaces such as the freshwater : saltwater interface is desirable (Goldberg, 1975).

The study of metal ion speciation in aqueous systems is characterized by contradiction. Coordination chemists have been able to derive elaborate equilibrium dynamics models which predict the behavior of metals in solution (Stumm and

Morgan, 1970). However, organic complexation which is predicted to be insignificant by equilibrium models is demonstrable by experimental methods. Authors questioning the importance of organic speciation of metals on the basis of equilibrium models or laboratory studies with model systems include Stumm and Morgan (1970), Duursma and Sevenhuysen (1966), and Pittwell (1974).

The experimental evidence for organic complexation is presented using a variety of techniques. In freshwater, gel filtration chromatography (Means, Crerar, and Amster, 1977) and dialysis or ultrafiltration (Benes and Steinnes, 1974; Giesy, 1976; Hart and Davies, 1977), demonstrated that both Fe and Co are associated with high molecular weight (organic) fractions. Electrochemical techniques have been applied by a number of workers (Chau and Lum-Shue-Chan, 1974; Chau, Gachter, and Lum-Shue-Chan, 1974; Ramamoorthy and Kushner, 1975; Shuman and Woodward, 1977) demonstrating the occurrence of high stability (organic) complexes. The techniques of gel filtration and electrochemistry have been applied together by Barsdate and Matson (1967). A five part analytical scheme to differentiate free, carbonate, cyanide, humate, and amino acid complexed Cu was developed (Stiff, 1971). A firmer theoretical basis for the occurrence of significant organic speciation in freshwater has been developed (Gardiner, 1974; Reuter and Perdue, 1977). In seawater, organic complexation of metals has been demonstrated by dialysis (Hood, 1967), organic extractability (Slowey, Jeffrey, and Hood, 1967; Williams, 1969; Alexander and Corcoran, 1967), bioassay (Davey, Morgan, and

Erickson, 1973), chelating resin chromatography (Stolzberg and Rosin, 1977), and by techniques combining electrochemistry, chelating resin chromatography (Duinker and Kramer, 1977) and ultraviolet ashing of organic matter (Batley and Florence, 1976). The use of equilibrium models of seawater metal ion speciation has been extended to include synthetic strong chelating agents, such as EDTA (Jackson and Morgan, 1978). Their treatment indicates that such chelating agents dominate the complexation of metals such as Cu. The organic fraction of seawater is still too undefined to apply such models.

The reactions of metals at the freshwater/seawater interface are a complex pattern of precipitation and remobilization reactions (Turekian, 1977). As freshwaters, low in salts and slightly acid in pH become increasingly diluted by saline and alkaline seawater, metals, both as dissolved species and as suspended colloids and particles, undergo precipitation reactions leading to the formation of flocculant materials which act as coprecipitants for a number of metals (Helz, Huggett, and Hill, 1975; Turekian, 1977). At the sediment/water interface of estuarine sediments, oxidative processes, perhaps biologically mediated, lead to the remobilization of trace metals into the water column (Turekian, 1977).

Inorganic constituents of seawater must exert a profound influence on the organisms of the oceans. One of the major advances of marine geochemistry is the recognition that conversely marine organisms have a profound influence upon the composition of seawater. This concept was proposed by Redfield (1958; Redfield, Ketchum, and Richards, 1963), indicating that

the ratios of the concentrations of the major nutrient elements are controlled by biological uptake fixing the ratio of N:P at 15:1, similar to the ratio of the elements in protoplasm (16:1).

Similar processes must occur for the trace metal components of seawater. Trudinger and Bubela (1967) have reviewed microorganisms as biogeochemical agents emphasizing the two way relationship between organism and environment. Marine organisms are capable of accumulating elements from their environment, often by four or more orders of magnitude (Bowen, 1966; Passman, 1977). Phillips (1977) has reviewed the use of accumulator organisms as indicators of metal pollution. The technique was based on the finding that the accumulation of the metal remained constant over a range of environmental metal concentrations (Morris and Bale, 1975). This gave a final concentration of metal in the organism which integrated the metal concentrations to which it was exposed. Microorganisms are capable of near complete uptake of metals from their growth medium in laboratory studies (Tornabene and Edwards, 1972) and in industrial effluents (Ilyaletdinov, Enker, and Yakubovske, 1977).

Several reports have appeared in recent years indicating that biological activity in seawater modifies metal concentrations in the water column. Norris (1971) reported variations in the dissolved and particulate concentrations of Zn, Cu, and Mn in seawater which correlated with a diatom bloom. Bender and Gagner (1976) have reported decreased concentrations of Cu, Cd, and Ni in surface waters of the Sargasso Sea relative to deep waters of the same area. They ascribed the

lower abundance of surface waters to biological uptake followed by sinking of organisms and derived detritis to lower depths and remobilization of the metals during mineralization, leading to enrichment of the aphotic zone by metals relative to the surface. A similar observation was reported by Boyle, Sclater, and Edmond (1976) who found that on a series of Geosecs stations in the Pacific, Cd correlated with phosphate showing similar profiles: low at the surface, increasing at the compensation depth, and stable below. This was interpreted as indicating that both are taken up by phytoplankton during primary production and released at depth by mineralization. Topping and Windom (1977), using the large scale environmental enclosure CEPEX system, found rapid encorporation of Cu into particulates (largely fecal pellets) which were lost from the water column.

Ni as an Environmental Hazard

Ni is one of the major constituents of the earth, as the planetary core is composed of an Fe-Ni mixture. However, its composition in the lighter crust is estimated at only 0.008 % (Anonymous, 1975). Ni concentrations are highest in basalts and other igneous rocks and lowest in sedimentary rocks. Biogenic carbonaceous minerals, coal, oil, and asphalts, are often high in Ni (Bowen, 1966). River transport of Ni is estimated at 1×10^4 ton/yr and ocean input due to washout from the atmosphere may be three fold greater (Anonymous, 1975). The source of atmospheric particulate Ni is believed to be largely from combustion of fossil fuels

(Lagerwerff and Specht, 1970; Merline, 1971; Schroeder, 1970). Ruhling and Tyler (1968) have documented a rise in the Ni content of soils relative to pre-1920 samples. Seawater baseline Ni values have been established at 1.2 ppb (2.0×10^{8}M) (Chester and Stoner, 1973) and sediments are estimated at 30 - 50 ppm (Phillips, 1977). World production of Ni is estimated to be 7×10^{5} tons, ten fold or greater than the natural Ni input to the oceans. The potential for pollution by this metal is high, particularly in view of the estimation that of seven major toxic metals, three, Ni, Cd, and Pb, are considered to represent major hazards to man (Schroeder, 1970). Wood (1975) included Ni among those metals which are "very toxic and relatively accessible". In addition, the 1971 Council on Environmental Quality included Ni among 14 potentially dangerous elements (Anonymous, 1971).

Ni has been the subject of an extensive review of medical and biological effects and of its role as an environmental pollutant (Anonymous, 1975). Ni was recognized to be of medical significance as early as 1826. While significant quantities of Ni are found in foods (grains 0-35 ppm, vegetables 0-5 ppm, tea 7.6 ppm, gelatin 4.5 ppm), normal exposures are harmless. However, a number of deleterious effects have been attributed to excessive doses or to Ni in unusual forms. Ni has been linked repeatedly to carcinogenesis (Goldblatt, 1958; Gilman, 1962; Sundermann, 1963; Baumslag, Keen, and Petering, 1971; Doll, Morgan and Speizer, 1971). Ni in the form of nickel carbonyl and as a variety of salts is a hazardous material, a "recognized carcinogen" responsible for pulmonary

and nasal neoplasms (Sax, 1975). Ni carcinogenesis may be related to the metal induced infidelity of DNA polymerase (Sirover and Loeb, 1976). Nickel carbonyl is a highly toxic gas and in addition to its carcinogenic properties is the cause of a severe pulmonary edema in exposed individuals (Anonymous, 1975). Nickel carbonyl poisoning was implicated briefly in the 1976 Legionnaire's disease outbreak following detection of apparent abnormally high levels of Ni in autopsy specimens. However, later findings showed these values were manipulative artifacts (Chen, Francisco, and Miller, 1977).

Metal Metabolism

An extensive literature exists on the uptake of trace metals of which only uptake by microorganisms will be reviewed here, starting with the essential trace metal: Fe. As early as 1932, Burke, Lineweaver, and Horner recognized growth stimulation due to Fe was mediated by Fe chelating agents. More recent work has demonstrated that Fe was transported into microbial cells by a highly specific system mediated by high stability constant Fe-specific chelating agents, termed siderophores. Major reviews include Spiro and Saltman (1969), Hutner (1972), Lankford (1973), Rogers and Neilands (1973), and Neilands (1974). It has been proposed by Hutner (1972) that all bacteria produced or required siderophores, an hypothesis which has been extended to include all organisms (Neilands, 1974). Siderophores can act not only as transport factors but also as antibiotics (Woodruff and Miller, 1963), presumably when the compound was bound to membrane receptors

but was non-functional. Chemically, siderophores are of two classes, both of low molecular weight: the hydroxamic acids, such as ferrichrome, and phenolates, such as enterochelin (Rodgers and Neilands, 1973). The recognition that siderophores bind to cells at highly specific sites has led to the use of these systems as probes of membrane structure (Pugsley and Reeves, 1976). Siderophores occur in aquatic environments (Lange, 1974; Estep, Armstrong, and Van Baalen, 1975), and producer organisms have been isolated from marine sources (Gonye and Carpenter, 1974). Such iron transport agents have been proposed repeatedly as a controlling influence on marine primary productivity (Lewin and Chen-hong Chen, 1971; Barber and Ryther, 1969) and as a triggering agent in dinoflagellate blooms (Martin and Martin, 1974). These agents were first detected and quantified by the research group under Lankford (Lankford, Kustoff, and Sergeant, 1957; Lankford et al., 1966; Byers, Powell, and Landford, 1967; Lankford, 1973) on the basis of an inoculum size-dependent lag phase phenomenon observed in bacteria of genera Bacillus and Arthrobacter. This lag was abolished by the addition of exogenous siderophore or by the production of siderophore by the cell.

The aspects of the uptake of other trace metals, both nutritive and toxic, have been studied less thoroughly than Fe but considerable data has been published. A general review of the topic has been published by Hutner (1972). In some cases metal-specific transport agents have been implicated in uptake processes, as in the uptake of Mn (Haavik, 1975; 1975a) by Bacillus licheniformis. This was mediated by bacitracin,

an antibiotic. The uptake of Mo by Bacillus thuringensis (Ketchum and Owens, 1975) and B. subtilis (Scribner et al., 1975) were mediated by low molecular weight peptide compounds. However, the majority of metal uptake systems occurred by cell bound systems or have not been investigated sufficiently to determine the role of soluble agents.

Uptake of Ni has been studied in a number of systems. E. coli adsorbed Ni at a site active in the adsorption of Cu and Co (Katayama, 1961). Bacillus megaterium took up Ni, Zn, and Co but not Cu and Cd by the same pathway which translocated Mg (Webb, 1970). B. subtilis cell walls accumulated a variety of metals including Ni (Beveridge and Murray, 1976; Beveridge et al., 1976). Jones et al. (1976) found that the dissimilatory sulfate-reducing bacteria took up a variety of trace metals, perhaps depositing them as intracellular electron dense particles though Ni was taken up less avidly than other trace metals. The uptake of seven trace metals including Ni was studied in six terrestrial and two marine bacteria in complex and defined media by Jones, Royle, and Murray (1976). Cobet (1968) found that a Ni-tolerant bacterium took up Ni from a Ni-containing growth medium with maximal relative uptake at the maximum Ni concentration allowing growth. A specific system for Ni assimilation has been found in the facultatively autotrophic, hydrogen-oxidizing bacteria which have a specific requirement for Ni (Bartha and Ordal, 1965; Bartha and Atlas, 1972; Tabillion and Kaltwasser, 1977).

Considerable work has been done on the uptake of Ni in the yeast Saccharomyces cerevisiae. Ni was bound by a

system which also binds Co and UO_2^+ and was related to glucose vectorial transport (Van Steveninck and Boojj, 1964; Van Steveninck and Rothstein, 1965). Later work indicated that transport of Ni, Co, and Zn was an energy dependent process linked to glycolysis occurring as a Na/K linked antiport system which functioned to transport Mg and Mn (Fuhrmann and Rothstein, 1968). Sivarama Sastry et al. (1962) found the toxicity of Ni, Co, and Zn was related to uptake of the metals by an Fe/Mg antagonized transport system. Okamoto et al. (1977) studied the uptake of a variety of heavy metals including Ni in a hyper-Cu tolerant fungus, Penicillium ochrochloron, finding a direct relationship between metal uptake and external metal concentration. A kinetic analysis of the uptake of radio-nickel by the diatom, Phaeodactylum trichornutum was performed by Skaar, Rystad, and Jensen (1974). Uptake of Ni was energy dependent and was increased strongly by the addition of phosphate to the culture following a lag period.

The uptake of Zn has been studied by several investigators. Energy dependent uptake was found in the fungus Neocosmospora vasinfecta by Paton and Budd (1972), in Dunaliella tertiolecta by Parry and Hayward (1973), in Escherichia coli by Bucheder and Broda (1974), and in Candida utilis by Failla and Weinberg (1977), Failla (1976), Failla, Benedict, and Weinberg (1976). In Candida utilis Zn accumulation was not uniform but showed a well-defined cycle with Zn uptake bursts in lag and again in logarithmic phases. By contrast, Cushing and Watson (1968) found Zn uptake in

phytoplankton to be non-energy dependent with higher uptake in killed than in live cells. Uptake of Zn by two metal tolerant marine bacteria under the influence of toxic levels of the metal was studied by Jones, Royle, and Murray (1976). They found maximum uptake of Zn at concentrations that were just sub-toxic. Energy dependence of uptake was not addressed.

The possible role of bacitracin in Mn transport has been mentioned. A specific energy dependent Mn uptake system has been detected in Escherichia coli (Silver and Kralovic, 1969; Silver, Johnseine, and King, 1970; Bhattacharyya, 1970), and during sporulation of Bacillus subtilis (Silver et al., 1974; Scribner et al., 1975).

Uptake of Cu by Neurospora crassa was reported by Sommers (1963). During sporulation of Bacillus megaterium, normally low Cu uptake showed a sharp peak coincident with a Cu requirement for sporulation (Krueger and Kolodzeij, 1976). Uptake in Saccharomyces cerevisiae was stimulated strongly by glucose (Ross, 1977). The stimulation of Cu uptake was great enough that Cu concentrations which were non-toxic to starved cells became toxic upon glucose addition.

Uptake of Hg has been studied in three phytoplankton: Dunaliella tertiolecta (Davies, 1976), Chaetoceros costatum (Glooschenko, 1969), and Pediastrum boryanum (Richardson, Millington, and Milns, 1975). Those authors concluded that Hg uptake was non-energy dependent (passive) and differences in uptake rates were correlated with Hg tolerance. The mechanism of Co uptake has been studied in Neurospora crassa indicating that translocation of Co into the cell was energy

dependent (Venkateswerlu and Sivarama Sastry, 1970). Co and Cd uptake in Saccharomyces cerevisiae was also energy dependent (Norris and Kelley, 1977). The uptake of these metals was presumably by a generalized metal uptake system of limited specificity. The uptake of free but not of chelated Cd by the diatom Phaeodactylum tricornutum was by passive adsorption (Crossa, 1976). Ag was adsorbed passively by the cell walls and membranes of Candida utilis (Golubovich, Khovrychev, and Rabotnova, 1976). The transuranium fission product ^{241}Am was assimilated by a variety of freshwater algae and bacteria (Giesy and Paine, 1976; 1977). The uptake of Pb by Micrococcus luteus and an Azotobacter species was studied by Tornabene and Edwards (1972). They reported startingly high accumulations of metal, up to 30 % dry weight of cells. Further growth would occur at Pb concentrations up to 2500 ppm in the growth medium.

A composite mechanism of non-ionophore mediated metal uptake can be derived. The first phase which occurs both in passive or active modes is the adsorption of metal cations to anionic exchange sites on the cell wall and membranes. In passive uptake, this is followed by a slow non-energy dependent diffusion of metal ions to complexing sites within the cell. In active uptake, initial passive adsorption is followed by energy dependent translocation of the metal to the cell interior. The phases of uptake were separated by the use of time course studies (passive uptake is very rapid, active translocation is slower), by metabolic inhibitors (passive translocation is uneffected, active transport is inhibited), and by uptake

capacity (passive uptake shows low saturation, active uptake shows much higher saturation concentrations).

Indirect evidence for a metabolic role of Ni from studies of its association with a variety of macromolecules was indicated. Ni was associated with nucleic acids (Wacker and Vallee, 1959). Ni and other transition metals stabilized the secondary structure of tobacco mosaic virus (TMV) RNA, though metal-treated-TMV RNA lost infectivity more quickly than controls (Huff et al., 1964). A Ni-containing α-2-macroglobin has been isolated from rabbit serum (Nomato, Neeley, and Sunderman, 1971). While no enzyme was activated specifically by Ni, a variety of enzymes were activated by this metal to a greater or lesser extent, such as: Jack bean arginolytic enzyme (Stock, Perkins, and Hellerman, 1938), phosphomonoesterase (Neuman, 1949), enolase (Wold and Ballon, 1951), carboxypeptidase (Coleman and Vallee, 1960), ribonucleic acid polymerase (Novello and Stirpe, 1969), arginase (Munakata, Niina, and Ueda, 1976), and isocitrate dehydrogenase (O'Leary and Limburg, 1977). A second line of indirect evidence for Ni essentiality has been proposed by authors who have noted a reproducible, slight stimulation of growth or yield at metal concentrations less than those at which toxicity was noted (Gonye and Jones, 1973; Cobet, 1968). However, this phenomenon has been noted by Lamanna and Malette (1965) as a typical feature of many toxic agents and has been termed the Arndt-Schultz Law. They proposed the phenomenon was due to disruption of allosteric control of metabolic pathways leading to more rapid, but uncontrolled, growth.

Direct evidence for a metabolic role of Ni was limited, though recent evidence has indicated a more widespread role. The first evidence of a metabolic role for Ni was found in two strains of the facultative hydrogen-oxidizing bacteria of genus Hydrogenomonas (Alcaligenes eutrophus). These bacteria had no requirement for Ni when grown heterotrophically, but for autotrophic growth on hydrogen the bacteria required 1.5×10^{-7}M Ni (Bartha and Ordal, 1965). The role was in carbon dioxide fixation rather than in hydrogen oxidation steps of metabolism (Hutner, 1972). These findings were questioned by Repaske and Repaske (1976) who studied the nutritional requirements of Alcaligenes eutrophus without finding an obligate requirement for Ni, though $1 - 2 \times 10^{-7}$M Ni was definitely stimulatory. Bartha and Atlas (1972) and Tabillion and Kaltwasser (1977) have studied the assimilation of Ni by this organism finding it possessed a specific, energy dependent transport system capable of near quantitative scavenging of the metal from dilute solution. Bertrand and DeWolfe (1973) found uptake of Ni by legume-Rhizobium symbiont nodules, improved in vitro growth of Rhizobium sp. at low concentrations of Ni, and increased numbers of active nodules on legumes grown in Ni-enriched soil. They proposed that Ni was required nutritionally for Rhizobium and proposed the use of 25 g/hectare of Ni as a nutritional supplement for leguminous crops.

Recently, evidence has been developed that Ni was an essential trace nutrient for higher vertebrates. Nielson and Sauberlich (1970) found that chicks grown on a diet deficient

in Ni (less than 0.08 ppm Ni) compared to controls fed the same diet plus Ni (5 ppm) showed multiple pathologies of bones, joints, and skin. Nielson and Ollerich (1974) extended these findings to include degenerative changes in hepatic metabolism and histology in the chick and similar pathologies in the rat when fed low Ni diets. The hepatic metabolism of Ni-starved rats was studied by Kirchgessner and Schnegg (1976) who demonstrated additional metabolic lesions in Ni-starved animals as well as decreased growth, reduced hematocrit and hemoglobin, and abnormal iron absorption.

The study of the nutritonal role of a metal required in very low concentration, but almost universally present as a trace contaminant, as was Ni, presents profound procedural problems. Reagent grade chemicals typically contain ppm levels of Ni sufficient to meet the nutritional demands of Ni. As techniques improve and more organisms are studied, a specific, widespread metabolic role for this metal may be indicated.

It is a paradox of trace metal nutrition that a metal which is essential or stimulatory at a given low concentration is toxic at some higher concentration, often only an order of magnitude (or less) greater than the nutritional optimum. Bowen (1966) described this pattern as an absence of growth at some low concentration followed by a rising curve through ranges of severe and slight deficiency, a plateau of growth in the range of optimal and luxury concentration, and finally a falling curve through ranges of first toxicity, then lethality. The demonstration of the toxic effects of a trace element is simpler than its essentiality. The optimum, luxury consumption,

and toxicity ranges, but not the deficient range were known for Ni until recently. It is likely that for a number of other heavy metals such as Cd, Pb, and Hg, further study will reveal the lower ranges of their action.

Minute amounts of metals may be required by an organism as calculated from knowledge of the size of bacteria. A coccoid bacterium 0.5 μm in diameter would have a volume of 6×10^{-14} ml. A typical protein molecule has a volume of 5×10^{-20} ml. Thus a cell of 10 % dry matter if composed of 50 % protein, 50 % other macromolecules would be comprised of about 50,000 protein molecules. It is reasonable to presume that many enzymes are present in 10 or fewer copies per cell. If each enzyme required only a single ion of Hg, a culture of 10^9 bacteria/ml, able to quantitatively scavenge the metal from solution could make do on a 10^{-11}M solution of Hg (2 ng/liter). Natural bacterial populations being far lower in density would require even lower amounts of a metal catalyst.

Metal Toxicity

The toxicity of elements is the subject of a substantial literature reviewed by Bowen (1966). This review is restricted to aspects of transition metal toxicity, particularly that caused by Ni and closely related metals: Co, Cu, Zn, and Cd. There were wide differences in the quantitative toxicity of different metals in a given system expressed in a toxicity series which ranks the relative potency of the elements studied. Reported series include those of:

Eisenberg (1918, cited in Porter, 1948) in bacteria:

$Ag > Hg > Pt > Au > Co > Cd > Ni > Zr > Tl > Cu > Pb > Nd > Al > Be > Ti > Fe^{3+} > Zn > U > Cr > Y > Fe^{2+} > Th > Ce > Mn > Ba > Ca > Sr > Mg > Li > NH_4 > K > Na$

Hotchkiss (1923) in E. coli:

$Hg > Cd > Ce = Al = Pb = Co > Fe^{2+} = Fe^{3+} = Cu = Zn > Ni = Sn = Tl > Mn > Ba > Ca > Mg > Sr > Li > NH_4 > Na > K$

Sommers (1960) in Botrytis fabae, a fungus:

$Os > Hg > Ag > Cu > Pd > Co > Ni > Cr > Pb > Yt > Tl > Be > Zn > Mn > Mg > Ca > Li > Sr > Ba > Na > K$

Avakyan (1967) in Rhodotorula sp., Torula sp., Hansenula sp., Serratia sp., Azotobacter sp., Pseudomonas sp., E. coli: $Ag > Hg > Co \geq Ni > Cd$

Vesper and Weidensaul (1975) in Rhizobium-soybean symbionts: $Cd > Ni > Cu > Zn$

Gachter (1976) in natural phytoplankton populations:

$Hg \geq Cu > Cd > Zn > Pb$

Okamoto et al. (1977) in Penicillium ochro-chloron, a fungus: $Hg >> Ni >> Tl > Co > Cr >> Pb = Cd = Fe >> Cu = Mn = Zn$

While there are differences in the relative placement of individual elements (Cd for instance), there is considerable agreement in the general order. This has led a number of workers to attempt to correlate toxicity with a variety of colligative properties of the elements. Suggested properties include:

The stability of bidentate chelates (Mellor and Maley, 1948): Pd >Cu >Ni >Co >Zn >Cd >Fe >Mn >Mg

Chelate stability (Irving and Williams, 1948):

Pd >Cu >Ni=Pb >Co=Zn >Cd=Fe >Mn >Hg

Stability of amino acid complexes or chelates (Albert, 1950): Cu >Ni >Zn >Co >Cd >Fe^{2+} >Mn >Mg >>Fe^{3+}

Electronegativity of the element (Danielli and Davies, 1949; Sommers, 1960):

Hg >Cu >Sn >Pb >Ni >Co >Cd >Fe >Zn >Mn >Mg >Ca >Sr >Ba

Stability of sulfide complexes (Bowen, 1966; Jones et al., 1976):

Hg >Cu >Pb >Cd >Co >Ni >Zn >Fe >Mn >Sn >Mg >Ca

Volume changes of proteins induced by metals (Katz and Roberson, 1976):

Pb >Cu >Zn >Cd >Ni >Co >Ca >Sr >Mg >K

While these properties tend to strongly resemble toxicity series, there are sufficient numbers of anomalies from any given set that there is little justification for invoking any one as the sole responsible property. It is likely that it is a synergism of several functions.

Bowen (1966) has defined six possible modes of elemental toxicity: 1. poisoning of enzymes, 2. action as an anti-metabolite, 3. precipitation or complexation with an essential metabolite, 4. catalysis of the decomposition of an essential metabolite, 5. binding to and modifying cell membrane

permeability, and 5. structural or electrochemical replacement of an essential element by a non-functional one. The induction of mutations (7) can also be added to this list (Venitt and Levy, 1974; Eichorn *et al.*, 1967; Sirover and Loeb, 1976). The first, fifth and seventh mechanisms are the most significant concerning the transition metals.

Poisoning of enzymes may occur by the complexation of cysteine sulfhydryl groups by metals (particularly Hg, Cu, and Ag) or chelation of amino, imido, and carbonyl groups leading to steric blocking of active sites, conformational changes, or disruption of catalytic groups (Mahler, 1960). Cell membrane effects are prominant with Ag, Cd, Cu, Hg, Pb, and U. These elements tend to bind with membrane components important in permeability regulation destroying permeability barriers and ion gradients. Rothstein (1959) has reviewed this area and presented criteria for differentiation of membrane vs cytoplasmic effects.

Mutational induction or potentiation has been reported for several metals, in bacterial systems: Mn in *E. coli* (Silver *et al.*, 1972), $CrO_4^{=}$ in *E. coli* (Venitt and Levy, 1974), As, Cd, Cr, Hg, Mn, and Mo in *B. subtilis* (Nishioka, 1975), Be, Mn and Ni in an *in vitro* system (Sirover and Loeb, 1976), and an undefined, probably metallic fraction from coal fly ash in *Salmonella typhimurium* (Chrisp, Fisher, and Lammert, 1978). It is not yet clear whether the effects are due to direct action on the nucleic acid or to infidelity of the polymerase due to metal binding to the enzyme.

A number of reports (Abelson and Aldous, 1950; Silver et al., 1972; Laborey and Lavollay, 1977; Marsh, 1945) indicate an antagonistic effect between Mg and several toxic metals. This evidence implied that the metals were acting as anti-metabolites of Mg. However, it is more probable that the effect was due to decreased binding of the metals to uptake sites rather than a direct metabolic replacement. Zn has also been reported to be antagonistic to Ni in Chlorella (Upitis, Pakalne, and Nollendorf, 1973).

Sadler and Trudinger (1967) have reviewed microbial metal toxicity; and Avakyan (1974) has published an extensive review of heavy metal toxicity in microorganisms giving an excellent entry into the Soviet literature. Of microorganisms, the viruses have been the subject of little research into heavy metal toxicity effects. Two references were found, those of: Groupe, Engle and Gaffney (1955) on the inhibition of influenza virus by free and chelated Co, and Mitchell (1971) on the die-off of phage in seawater due to metals.

By contrast, the literature on the toxicity of heavy metals to bacteria is immense. A computer search has produced 60+ references directly related to bacterial metal toxicity. A conservative estimate would place the total well over 200. The effects of metoxy-anions, Hg, and non-transition metals were excluded. Most reports of metal toxicity in bacteria have examined a single bacterial species, or small group of closely related species, although frequently the effects of several metals may be reported. Avakyan (1967) studied the effect of five metals (Co, Cd, Ni, Ag, and Hg) on 31 strains of

microorganisms finding yeasts most tolerant, followed by Serratia, Azotobacter, and Pseudomonas, with E. coli most sensitive. Babich and Stotzky (1977) compared the toxicity of Cd to a variety of microorganisms including gram-positive and negative-bacteria, actinomycetes, and fungi. Actinomycetes were more tolerant than eubacteria, and gram-negative more tolerant than gram positive bacteria. Fungi varied widely in tolerance. Ag inhibited Mycobacterium sp. at 10 µg/liter, while Candida utilis and Pseudomonas sp. were inhibited at 60 - 100 µg/liter, and Aspergillus niger was inhibited at 500 µg/liter (Golubovich, 1974).

The toxicity of Ni to bacteria has been recognized at least since 1893 (Avakyan, 1974) by the occurrence of inhibition zones surrounding metal disks on agar. Hotchkiss (1923) studied the quantitative toxicity of a variety of metals including Ni to E. coli. Ni gave total inhibition at 5×10^{-3}M in a 1 % peptone medium. Abelson and Aldous (1950), also working with E. coli, found 3×10^{-7}M Ni (20 µg/liter) was toxic in a glucose - mineral salts medium low in Mg, while at 200 mg/liter Mg^{++}, 1×10^{-5}M (600 µg/liter) Ni was required to show toxicity. The coordination of the flagellar tuft of Spirillum volutans was disrupted by Ni, Cu, and Mg as well as the inhibitors chloral hydrate and phenol (Kreig, Tomelty, and Wells, 1967). Ni inhibited RNA synthesis to a greater extent than protein synthesis in E. coli (Blundel and Wild, 1969). Cd, Zn, Cu, and Mo had a similar activity. By contrast, Co showed greater protein synthesis inhibition than RNA synthesis.

The study of the effect of Ni on a marine bacterium has been the subject of a series of publications (Wirsen, 1966; Cobet, 1968; Cobet, Wirsen, and Jones, 1970; Cobet *et al.*, 1971; Gonye, 1972). The organism, isolated during a study of the prevalence of Ni-tolerant marine bacteria, formed abnormally large, plasmolysed cells under metal stress (Cobet, 1968). Growth yield and rate decreased with increasing metal concentration in the range of 1 to 5 X 10^{-4}M Ni in a peptone - yeast extract broth. The toxic Ni concentration was inoculum dependent. Under conditions of Ni stress, the cells leaked protein and nucleic acid materials into their milieu (Jones, 1970). The site of Ni action was the cell membrane/wall (Gonye, 1972).

Vesper and Weidensaul (1975) found that nitrogen fixation by *Rhizobium*-soybean symbionts and *in vitro* growth of *Rhizobium* was inhibited by Ni, Cd, Co, and Zn, though the order of the toxicity series was different for the symbionts than for the free-living bacteria. The lipolytic activity of a *Mycobacterium* and an *Actinomyces* sp. was inhibited by Ni and a variety of other metal cations (Lebedeva, Volmova, and Ruban, 1976). Arcuri and Ehrlich (1977) studied the effects of hydrostatic pressure and heavy metal toxicity (Mn, Cu, Co, and Ni) on three deep sea bacteria. In most cases, pressure and metals acted synergistically implying the potential for greater metal toxicity in the deeps than at the surface.

The toxicity of Cu to bacteria also has been recognized. The 1923 studies of Hotchkiss (Cu toxic to *E. coli* at 1 X 10^{-4}M in 1 % peptone) along with Winslow and Haywood (1931) mark the

beginnings of its quantitative study. Waksman, Johnstone, and Carey (1943) isolated bacteria from seawater capable of growth at 1 X 10^{-3}M Cu on a 0.1 % peptone / 0.1 % yeast extract medium. These bacteria formed characteristic brownish colonies on Cu-containing media and were not recoverable from freshwater. The growth of Mycobacterium phlei was inhibited by 250 µg/liter (4 X 10^{-6}M) Cu in a glucose - mineral salts medium (Pratt, 1953). The addition of citrate or α-amino acids (chelating agents) reversed toxicity. Cu frequently has been reported to induce small colony variants in both bacteria and fungi. These are stable, apparently mutational events which led to the loss of respiratory metabolism, and/or loss of fermentation ability of various sugars, and have been reported in Bacillus (Weed, 1963) and E. coli (Weed and Longfellow, 1954; Hisch, 1961). A Cu-tolerant marine isolate, Pseudomonas cuprodurans (P. putida) was studied by McCarthy (1971) who found increased lag time, lower temperature optimum, alterations in protein and nucleic acid metabolism, and respiratory deficiency in Cu-stressed cells. The sensitive region of the respiratory chain was the flavo-protein/quinone level. The respiratory deficiency induced in this bacterium is not stable and thus probably not mutational.

In bacteria (E. coli and Enterobacter aerogenes) and in phytoplankton there have been several reports of inhibition due to low concentrations of Cu, as from tin-plated stills, present in defined composition growth media (Hofstein, 1962; Burke and McVeigh, 1967; MacLeod, Kua, and Gelinas, 1967). Reported toxic levels range from 1 to 15 X 10^{-7}M Cu.

Cu-complexing organic matter suppressed Cu toxicity (MacLeod, Kua, and Gelinas, 1967). In a medium of 0.025 % organic matter (peptones and acetate), Starr and Jones (1957) reported that marine bacteria from a variety of sources were uninhibited at up to 250 µg/liter Cu (4×10^{-6}M). The majority grew in up to 50 mg/liter (8×10^{-4}M). The importance of the metal-complexing ability of the medium has been emphasized by Sadler and Trudinger (1967) who found Proteus to be inhibited at a 1000-fold lower Cu concentration in a glucose - mineral salts medium as compared to a peptone medium. With an unidentified Pseudomonas isolate in a glucose - mineral salts medium, inhibitory concentrations of Cu (10^{-4}M) stopped growth and division but not metabolic activity. Cu inhibited cells adapted ultimately to Cu showing increased respiration under Cu stress compared to control cells and reduced uptake of Cu. Cu caused a rod to coccus cell-shape shift and inhibition of cell motility. Milanovich, Wilson, and Yeh (1975) found that the toxicity of Cu (6×10^{-6}M) to E. coli in a glucose - mineral salts medium was abolished by addition of equimolar EDTA or humic acid (a natural chelating agent - "gelbstoff"). Beswick et al. (1976) found the toxicity of Cu to E. coli greater under anaerobic than aerobic conditions due to reduction of Cu^{++} to the more toxic Cu^{+} form.

The study of the toxicity of Co also has its historical roots in the studies of Hotchkiss (1923) (5×10^{-3}M in 1 % peptone medium), Winslow and Haywood (1931), and Abelson and Aldous (1949). Schade (1949) found Proteus vulgaris to be 400-fold more tolerant of Co in nutrient broth (2×10^{-3}M) than

in glucose - mineral salts medium (5×10^{-6}M). Histidine and cysteine reversed Co inhibition at a 2:1 molar ratio. Under anaerobic conditions Co was less toxic than under aerobic conditions (Beswick et al., 1976). Blundell and Wild (1969) found that during inhibition of E. coli by Co, there was accumulation of RNA in cells, perhaps ribosome precursors.

In the above discussion of metal toxicity, frequent mention has been made of the amelioration of metal toxicity by metal complexing agents. However, metal chelate compounds need not exert less toxicity than the free metal, indeed the chelate may be more toxic than its corresponding free metal or chelating agent. In addition to the hydroxamate anti-metabolite antibiotics already mentioned, a series of Cu-chelate antibiotics from Pseudomonas reptilovora has been reported (Del Rio et al., 1972). Weinberg (1953) published an early review of the role of metal chelation in the activity of anti-biotics. 8-hydroxyquinoline (oxine), which is widely used as a preservative, was most active as a 1:1 chelate with various metals, such as Cu (Martell and Calvin, 1952). This can give rise to effects in which a plot of growth versus metal:chelate ratio showed multiple minima, one at free metal, and one or more at ratios of 1:1, 2:1, 1:2, etc. Evidence for such effects was the "liesegang ring" phenomana (Feeney, Petersen, and Sahinkaya, 1957; Johnson, Carver, and Harryman, 1942) in which metals or chelating agents diffusing outward from the center of a bacteria-seeded agar plate containing chelating agents or metals gave rise to multiple rings of stimulated growth separated by zones of inhibition. However, using a

similar diffusion plate assay for metal tolerance in Azotobacter, Den Dooren De Jong (1971) reported no growth rings. Working in peptone media with several enteric bacteria, Annear and Mee (1976) reported two inhibitory ranges of Ag concentration, 5-20 mg/liter and greater than 50 mg/liter, analogous to the liesgang phenomenon.

In recent years a number of authors have attempted to investigate the role of metals in natural environments. Gonye and Jones (1973) studied the number and distribution of Ni tolerant bacteria in the open ocean. Consistantly higher counts were obtained on plating media containing non-toxic (1×10^{-5}M) levels of Ni as compared to Ni-free controls. Higher proportional Ni-resistant populations were obtained in open ocean waters as compared to coastal waters. Albright and Wilson (1974), using heterotrophic uptake techniques, found significant reduction in the activity of natural freshwater populations at 0.1 µg/liter Ag, 100 µg/liter Hg, 50 µg/liter Zn, and 10 µg/liter Cu. Walker and Colwell (1976) found that low concentrations of Hg inhibited the mineralization of petroleum by 11 oil-utilizing isolates from Chesapeake Bay. Mills and Colwell (1977) found that rates of nitrification and glucose oxidation by natural populations were inhibited by 100 mg/liter concentrations of Cr, Cd, Co and Pb, and 10 mg/liter Hg. These same metal concentrations in agar plating medium gave reduced numbers. Inhibition was less, and resistant numbers higher from a polluted area compared to unpolluted areas of Chesapeake Bay. The controlled ecosystem pollution experiment (CEPEX) study of the effects of low (10 and 50

µg/liter) additions of Cu to large scale environmental enclosures has found strong short-term stimulation of heterotrophic activity and bacterial counts following Cu pulse additions. Further, Cu-stressed enclosures developed populations resistent to Cu additions (Vaccaro, Azam, and Hodson, 1977). Watson and Waterbury (1969) and Truper (1969) have studied the bacterial populations of the hot, hypersaline metal rich waters of the Atlantis II and Discovery deeps of the Red Sea. The hottest and most dense layers (44-56°C, 20-35 % salinity) are sterile by a variety of cultural techniques.

The well known phenomenon of the die-off of freshwater bacteria in the oceans (Carlucci and Pramer, 1959; Mitchell, 1971; Jones, 1971), though ascribed to a wide variety of possible mechanisms, may be due to the toxic action of metals in seawater upon the metal sensitive freshwater bacteria. Evidence for the activity of metals has been presented by Carlucci, Scarpino, and Pramer (1961), Jones (1964), Moebus (1972), and Jones and Cobet (1975) by the observation that the survival of freshwater bacteria is extended by addition of small amounts of known chelating agents to seawater or autoclaving samples so that the metals are coprecipitated by $CaCO_3$ (Jones, 1967). Metal toxicity may also be implicated in the die-off of enteric viruses in the sea (Aken, Hill, and Clark, 1975).

Horsfall (1956) has reviewed the literature on metal toxicity in fungi, particularly those of agricultural significance. He traced the beginning of scientific study of the

toxicity of heavy metals to the use of Bordeaux mixture ($CuSO_4$) to control mildew of wine grapes in 1800.

Ross (1975) has reviewed heavy metal effects in both yeasts and mycelial fungi. Co, Mn, Zn, Ni, and Cu were taken up by yeasts and probably mycelial fungi had intracellular sites of action. By contrast, U ang Hg acted at the cell surface without penetration. The mycelial fungus Neurospora crassa has been studied with respect to its Ni, Co, and Zn inhibition (Sivarama Sastry et al., 1962; Venkateswerlu and Sivarama Sastry, 1973). They found the toxicity series Ni>Co>Zn. Metal toxicity was antagonized by Mg and Fe, apparently by reduced binding of the toxic metal. A Co-resistant substrain, derived by training onto increased concentrations of Co, was stable and gave increased resistance to Ni but unchanged or decreased tolerance of Cu and Zn. Aspergillus niger has been studied by Zlotchevskaya and Rabotnova (1968). They found that toxicity of Pb was modulated by the complexing capacity of the medium, and that detoxification of the Pb could be caused by the organism producing citrate which complexes Pb.

Considerable work has been reported by a Japanese group under the direction of Ashida (1965) on the toxicity of Cu to the yeast Saccharomyces cervesiae. However, much of this work was directed toward the mechanisms of adaptation and resistance to the metal, and will be discussed below. The induction of respiratory deficiency (Lindgren, Nagai, and Nagai, 1958) by Mn, Cu, Co, and Ni has been reported in S. cervesiae. These metals caused the stable loss of respiratory capability at

concentrations below those which killed the cells which might indicate mutagenesis. However, in addition to inhibition of respiratory activity, Ni would also partially inhibit fermentation at sublethal metal concentrations due to a specific sensitivity of the ethanol dehydrogenase enzyme to Ni (Fuhrmann and Rothstein, 1968). Cook (1973) found a differential effect of Fe, Cu, and Mn on respiration and fermentation of S. cervesiae, but fermentation was more sensitive than respiration. Ross (1977) found that the uptake, and thus the toxicity of Cu to S. cervesiae was potentiated by glucose. Resting cells which did not transport Cu were highly resistant, but were rapidly poisoned on the addition of glucose due to immediate induction of uptake.

A group of Russian workers have studied the toxic effects of Cu and Ag upon the yeast Candida utilis (Avakyan and Rabotnova, 1966; 1971; Golubovich and Rabotnova, 1974; Khourychev, Ivanova, and Taptykova, 1974; Petrova et al., 1976; Khourychev et al., 1977). Cu inhibited the cell by suppression of respiration, disruption of biosynthetic balances, and damage to the cell membrane, leading to cell leakage. Cu caused morphological changes. Analysis of the toxicity of Cu in relationship to its speciation in solution showed that the Cu was free or weakly complexed, while strongly complexed Cu was non-toxic. By contrast, Ag was up to 100-fold more toxic than Cu under identical conditions and did not cause morphological changes. Kinetic analysis of growth inhibition by Ag showed non-competitive inhibition up to a concentration above which the kinetic plot deviated from linearity. This phenomenon was

interpreted as indicating inhibition of a single key enzyme at low concentrations with multiple inhibitions above the break point (540 μg/liter in a mineral salts medium). The effect of Ag was studied with Cryptococcus albidus (Brown and Smith, 1976). No morphological effects due to Ag occurred, but the deposition of electron-dense, Ag-containing particles was found. These authors refer to unpublished results showing the formation of giant cells due to Cd. The effect of Zn upon Torulopsis homii was studied by Steenberger, Steenberger, and Weinberg (1969). At Zn concentrations from 10^{-5} to 10^{-4}M there was apparently normal growth followed by rapid die-off during stationary phase, apparently due to Zn-mediated suppression of secondary metabolism. Cu and Cd caused a similar effect.

The unicellular algae show wide variations in reported concentrations of inhibition by toxic metals. Part of this variation is due to differences in the complexing capacity of the growth media. On the other hand, there are real differences in the sensitivity of different algal strains to the same metal and of a single strain to different metals.

Den Dooren De Jong (1965) studied the toxicity of 49 metallic cations to the alga, Chlorella vulgaris. Co, Cu, and Ni had toxic levels from 4.2×10^{-6} to 2.9×10^{-5}M. In the related C. pyrenoidosa, Steeman-Nielsen and Kamp-Nielsen (1970) found 1×10^{-6}M Cu gave complete inhibition. At slightly lower Cu concentrations, growth was normal but occurred after a lag period. The addition of artificial chelators counteracted the toxicity of the metal as did medium in which cells had grown previously. This same research group (Steeman-Nielsen and

Wium-Andersen, 1970; 1971) compared the toxicity of Cu to C. pyrenoidosa and the diatom Nitzchia palea. In media free of chelators both were inhibited at Cu concentrations (c. 10 µg/liter) which are typical of coastal waters and only slightly greater than those in open ocean waters (2 µg/liter). This concentration was exceeded in single-distilled water (250 µg/liter), thus experiments were prepared with glass-redistilled water. The lag due to Cu in Chlorella was not seen in N. palea, but in the presence of Cu the diatom excreted organic matter which bound Cu, making the medium suitable for growth. The concentration of Cu which was inhibitory was dependent on the inoculum; low inocula were more sensitive than high inocula. Studying the same two algae, Wium-Andersen (1974) found that concentrations of Cr derived from chromic acid washed glassware were strongly inhibitory. However, Cr was less toxic than Cu by a factor of about 100.

Erickson (1972) reported that the Cu levels in unenriched seawater (0.7 - 1.1 µg/liter) were inhibitory to the diatom Thalassiosira pseudonana, but artificial or natural (organic detritus) chelators abolished Cu toxicity. Using the same organism, Sunda and Guillard (1976) found that the inhibition of growth rate and the Cu concentration of cells was proportional to the ion activity. The Cu concentration of ocean seawater in the absence of artificial or natural chelating agents was sufficient to inhibit the diatom. Gonyaulax tamarensis, the dinoflagellate responsible for paralytic shellfish poisoning in the North Atlantic region was inhibited at Cu ion activities of $10^{-9.7}$M compared to the seawater Cu

activity of $10^{-9.6}$M in the absence of organic chelation (Anderson and Morel, 1978). Organic chelating agents must be present before the dinoflagellate can multiply to bloom proportions. Using natural populations of phytoplankton, Gachter (1976) found that concentrations of Cu equal to natural levels were inhibitory and further that the metals (Hg, Cu, Cd, Zn, and Pb) acted synergistically leading to the occurrence of metal toxicity at metal concentrations lower than natural. This raised the possibility of natural phytoplankton populations normally being in an inhibited state.

Comparative studies of the heavy metal toxicity response of different species of phytoplankton have indicated that green algae are least sensitive, dinoflagellates are more sensitive, and diatoms are most sensitive (Kenis et al., 1973). Further, the more easily cultured diatom species, such as Phaeodactylum tricornutum, are far more metal-tolerant than the difficult to culture forms such as T. pseudonana (Jensen, Rystad, and Melsom, 1974; Braek and Jensen, 1976). Combining these areas of evidence, the easily cultured forms are so due to their resistance to the high background concentrations of metals in their media. This is borne out by the observations of Thomas and Seibert (1977) on the changes of phytoplankton populations in Cu treated ecosystem enclosures. The previously dominant, and difficult to culture centric diatoms were replaced by pennate diatoms, such as Nitzchia and Navicula, which are easily cultured.

Observations such as these have led to proposals that phytoplankton succession and productivity are linked to the

toxicity of metals and their complexation by organic matter (Provasoli, 1963; Johnston, 1964; Barber and Ryther, 1969; Yentsch et al., 1975). This contrasts with the theory presented above that metal nutrient availability mediated by iron chelating agents modulates productivity. The application of an equilibrium model by Jackson and Morgan (1978) indicated that the Fe availability hypothesis could not explain observed phytoplankton stimulation by chelating compounds. They concluded that Cu detoxification is the mechanism of phytoplankton growth stimulation. Davey, Morgan, and Erickson (1973) have developed an assay of the Cu complexation capacity of seawater based on the sensitivity of T. pseudonana to free ions of that metal.

By contrast, some evidence exists that the sole agent of metal toxicity is not free Cu ion; chelated ion may exhibit toxicity as well (Gachter, Lum-Shue-Chan, and Chau, 1973; Khobot'ev, Kapkov, and Zukhapze, 1969; Fangstrom, 1972). The previously cited works have in the main been performed with culture media low in chelating agents or in which chelation has been controlled as an experimental variable. A very large body of phytoplankton toxicity work exists in which metal toxicity has been measured in media having extremely high levels of chelating agents and in which the authors failed to take into account the metal complexing capacities of their experimental systems (Overnell, 1976).

An effect of toxic metals which has been reported from many microorganisms is the induction of abnormal cellular morphology, typically the formation of abnormally large cells. The distribution of morphology effects due to toxic metals is

spotty. Not all metals cause such effects, nor do different organisms show the same morphology due to the same metal. Such abnormally enlarged cells have been termed megalomorphs by Cobet, Wirsen, and Jones (1970). A commonly observed form of abnormal morphology is the induction of elongated filamentous cells without crosswalls, as by Pt and other periodic group VIII B elements (Rosenberg et al., 1967; Durig et al., 1976; Moore and Brubaker, 1976), by complexes of Co and Cd (Theodotou et al., 1976), and by Cu (Bubela, 1970). Identical filament forms can be induced by hydrostatic pressure (ZoBell and Cobet, 1964).

A different form of megalomorphy in which enlargement is in two or three dimensions, giving rise to swollen cells as opposed to filamentous cells, may also occur. Ni, at levels just subtoxic, caused extreme enlargement (500 fold or greater) of the cells of a Ni-tolerant marine bacterium due to uncoupling of growth and cell division (Cobet, Wirsen, and Jones, 1970; Cobet et al., 1971). A similar effect due to U has been reported by Tuovinen and Kelley (1974). An apparently identical effect is seen in the mitochondria of certain freshwater unicellular algae when poisoned by EDTA-chelated Cd (Silverberg, 1976). Metal-induced morphology changes have been reported in phytoplankton (Davies, 1976) and in yeasts (Khovrytchev et al., 1977), though the magnitude of the volume increase (2 - 4X) is much less than in the procaryotic forms.

Cells morphologically identical to those produced by Ni in A. marinus (Cobet, 1968) can be induced in E. coli K-12 by a combination of genetic mutations and UV irradiation, or

by antimetabolite (6-aminoinopenicillanic acid) treatment. The formation of amorphously enlarged cells, rather than filamentous forms is under genetic control in this system (Adler et al., 1968).

Metal Resistance

The review of Ashida (1965), presented an excellent summary of modes and mechanisms of metal resistance in all organisms. Ashida has defined two indices for the occurrence of resistance in an organism: 1. increased metal necessary to achieve the same effect, and/or 2. a reduced effect at the same concentration. He detailed nine possible mechanisms of resistance: 1. increase in the activity of an affected metabolic pathway; 2. induction of an antagonistic metabolic pathway; 3. increased synthesis of an inhibited enzyme; 4. decreased requirement for the product of an inhibited pathway; 5. modification in the enzyme yielding a decreased inhibitor affinity or increased substrate affinity; 6. destruction of the toxicant; 7. decreased cell permeability which excluded the toxicant; 8. production of a metal-binding agent; and 9. changes in the cytoplasm such that enzymes function in the presence of the metal. To this may be added: 7a. production of, or growth in, hyperacidic conditions, leading to charge exclusion of metal ions. Examples will be presented of the 6th, 7th, and 8th mechanism, as well as responses which might represent one or more of the other mechanisms. The review of Sadler and Trudinger (1967) should also be referred to for an excellent discussion of heavy metal resistance mechanisms,

particularly the differentiation of modification of the environment (mechanism 8 above) from modification of the cell, and somatic adaptation from mutation and selection.

The resistance to metals afforded by acidophilic or acidogenic growth can lead to organisms having the capacity to grow in nearly saturated concentrations of metals. This property is apparently widespread in the fungi but in the bacteria is restricted to the members of the genus Thiobacillus. Hyper-acidic environments are typical of waters draining pyritic ores. Ehrlich (1963) studied a mine drainage stream with pH 2.5, 800 ppm Cu and 1060 ppm Fe, finding Thiobacillus, Rhodotorula and Trichosporon yeasts, amoebae and flagellate protozoa. Booth and Mercer (1963) reported inhibition of Thiobacillus at 0.1 - 0.2 % Cu. The iron-oxidizing bacterium Thiobacillus ferrooxidans was resistant to Zn, Ni, Cu, Co, Mn, and Al at greater than 0.1 % concentrations (Tuovinen, Niemela, and Gyllenberg, 1971; Tuovinen and Kelly, 1974; Imai et al., 1975), although Ag, U, and metoxyanions of Mo, Te, Ag, and Se were much more toxic (5 to 100 mg/liter). Similar high tolerance concentrations were recorded during sulfur oxidation by this bacterium but during the oxidation of thiosulfate, metals were far more toxic (Co 5 mg/liter, Zn 600 mg/liter, Ni 150 mg/liter). Reports of acidophilic, hyper-metal tolerant fungi include Scytalidium sp. (Starkey and Waksman, 1943; Starkey, 1973), Penicillium nigrifaciens (Singh, 1977); and Penicillium ochro-chloron (Okamoto et al., 1977). Scytalidium grew in 1 M $CuSO_4$ at a pH of 2 to 3, but was inhibited by 4 X 10^{-3}M Cu at pH 7. Penicillium nigrifaciens was isolated from a saturated

solution of Cu-tartrate. At pH 2.8, its growth yield in 4 X 10^{-2}M Cu was as great as at pH 5.6 and 4 X 10^{-5}M Cu. Penicillium ochro-chloron in a glucose - mineral salts medium was uninhibited by saturated (10 %) Cu, Mn, and Zn, 2 % Pb, Cd, and Fe, 0.3 % Cr, 0.2 % Co, 100 mg/liter Ni, and 10 mg/liter Hg. The order of toxicity, especially the relatively high sensitivity to Ni, was quite distinct from that of Thiobacillus ferroxidans. These unusual metal tolerances were interpreted as cell wall/membrane-mediated phenomena in which the acidic conditions caused protonization of the anionic sites at which the toxic metals normally bind leading to a neutral or positively charged cell which electrostatically repels the metal cations. However, the data of Imai et al. (1975) indicated that there may also be unusual metal tolerance of enzymes, at least in Thiobacillus, as cell-free suspensions were capable of Fe^{2+} oxidation at metal concentrations as high as those tolerated by intact cells.

Foully (1976) has noted that bacteria in soil were uninhibited by Pb concentrations which were strongly inhibitory to soil bacteria in vitro. Babich and Stotzky (1977a, b) have found that the clay minerals montmorillonite and kaolinite act to protect a variety of bacteria and fungi from the toxicity of Cd. Clay minerals, such as those which occur naturally in soils, were capable of adsorbing and thus detoxifying heavy metals.

By contrast with the examples above, the majority of work in microbial metal resistance has been on systems in which the agent of resistance is either a modification of the

cell itself, or in which a product of cell metabolism acts to detoxify the metal internal or external to the cell. There are a large number of reports in which a mutational event, often due to the activity of the metal as a mutagen, leads to the stable aquisition of metal resistance in the resulting population. Minagawa (1957) reported the production of an RNA fraction by Cu-adapted Saccharomyces cervesiae cells which caused reduced Cu sensitivity of the parental strain. Antoine (1965) mapped a Cu resistance locus on Cu-adapted S. cervesiae. Silver et al. (1972) found Mn resistant mutations which mapped on the E. coli chromosome. Picket and Dean (1976) found that Cd-resistant strains of Klebsiella aerogenes showed simultaneous resistance to Zn and four antibiotics compared to the parental strain. Passage of domestic sewage cultures through increasing concentrations of Pb gave rise to Pb-resistant cultures of Escherichia, Klebsiella, Proteus, Serratia, and Achromobacter which also showed resistance to Cu, Hg, Ag, Cd, Bi, and As (Varma, Thomas, and Prasad, 1976). Tohoyama and Murayama (1977) induced Cd-resistance mutations by both training onto increasing concentrations of Cd and by ultra-violet mutagenesis. It is also possible for mutations to give decreased resistance to metals (Corwin et al., 1966; Ohta and Udaka, 1977).

R-factors (plasmids), the transmissable extrachromosomal genetic elements of bacteria which carry antibiotic resistance genes, are also frequently carriers of metal resistance genes. This was first reported by Smith (1967) who found that E. coli strains which carried antibiotic resistance plasmids showed coordinated transfer of antibiotic and

Hg resistance implying that the two were linked on a single genetic element. Ni and Co resistance factors were found. Other reports of plasmid linked metal resistance include: As, Pb, Cd, Hg, Bi, Sb, and Zn resistance in Staphylococcus aureus (Novick and Roth, 1968); Hg resistance in E. coli (Sommers, 1973); Te resistance in a number of bacteria (Sommers and Jacoby, 1977); Hg, Cd, and Ag resistance in Pseudomonas aeruginosa (Nakahara et al., 1977); and As and Cr resistance in Streptococcus lactis (Efstathiou and McKay, 1977).

If a toxic agent can not reach its site of action it will be ineffective. Decreased uptake of metal or cell permeability changes have been reported in a number of microorganisms. This mechanism has been implicated in the resistance of a variety of bacteria to Co and Ni (Webb, 1970). The Mg transport system, which also transports the toxic metals in the parental strains, had a much lower affinity for the toxic metals in the resistant strains derived from them. Chopra (1970) has found that a strain of Staphylococcus aureus which carried a Cd resistance plasmid showed decreased uptake of Cd. Dikanskaya (1971) proposed decreased permeability as the mechanism of Co resistance in yeast. Greenaway (1972) found reduced uptake of phenyl mercury by a resistant strain of the fungus Pyrenophora avenae. Mitra et al. (1975) found that somatic adaptation of E. coli to Cd occurred by a mechanism of cell wall alterations which prevented passage of Cd into the interior of the cell. Davies (1976) implicated this same mechanism in the resistance of the alga, Dunaliella tertiolecta,

to Hg, as did Foster (1977) in the resistance of a strain of Chlorella vulgaris to Cu.

Another important mechanism of resistance is the destruction of the metal, or more properly, its removal from the cell's environment. This can be achieved by the precipitation of the metal as a sparingly soluble form, such as a sulfide, or the conversion of the metal to a volatile form, such as the methylation of Hg. Cellular production of H_2S, which reacts with a variety of heavy metals giving extremely insoluble precipitates was reported in both the sulfate reducing bacteria and in fungi. Ashida and Nakamura (1954) and Ashida (1965) have reported the production of hydrogen sulfide by both yeasts and mycelial fungi which gave distinct resistance to Cu in the sulfide producer strains. Cu was deposited as intracellular granules. The dissimilatory sulfate-reducing bacteria such as the genus Desulfovibrio which produce large amounts of sulfide as a byproduct were rendered highly metal resistant by it (Sadler and Trudinger, 1967; Booth and Mercer, 1963; Vosjan and VanDerHoek, 1972).

The second mechanism of metal removal, that of conversion of the metal to a volatile form, has been documented extensively as a widespread mechanism of Hg-resistance in microorganisms. Savena and Howard (1977) reviewed the transformations of Hg, as well as As, Se, and Pb. This mechanism was found in plasmid-borne Hg resistance in E. coli and Pseudomonas sp., in which the volatile form was metallic (Sommers, 1973; Hayakawa, Kusaka, and Fukui, 1975). This mechanism was also found in various algae, including Dunaliella

(Betz, 1977) and Chlorella (Ben-Bassat et al., 1972; Ben-Bassat and Mayer, 1975; 1977). Volatilization of Pb, as tetramethyl lead, has been reported from a variety of gram-negative bacteria from lake sediments under anaerobic conditions (Wong, Chau, and Luxon, 1975).

The final mechanism of metal resistance is that of the detoxification of the metal either within the cell or in the surrounding medium by the production of metal-complexing agents which bind with the metal yielding a non-toxic form. This mechanism was invoked by Katayama (1961) to explain the resistance of an E. coli strain which took up more Co than its non-resistant parent strain. Khailov (1964) found that a variety of algae, including Dunaliella, Fucus, and Ascophyllum, excreted Cu-binding organic matter into their growth medium. Corpe (1975) studied the production of surface active polymers from marine bacteria which form primary films on surfaces. These polymers formed complexes with Fe, Cu, Pb, Co, Ni, and Zn. However, though these bacteria were somewhat metal tolerant, they did not form more or less of the polymers in the presence or absence of metals. Pelroy et al. (1977) found evidence for the production of Cd and Ni binding agents by a variety of bacteria and fungi isolated from soil. Costerton, Geesey and Cheng (1978) have reviewed the function of the "glycocalyx" of cells, the extended polysaccharide matrix which surrounds cells. They proposed that these polysaccharides acted as cation exchangers to shield the cell from toxic metals.

A metal resistance mechanism acting by the induction of low molecular weight peptide-binding agents appears

widespread in higher animals. These peptides, termed metallothionens, are found in hepatic and renal tissues, are induced by the metals which they bind, and are the major response of higher animals to toxic metals (Winge and Rajagoppian, 1972; Pulido, Kagi, and Vallee, 1966; Webb, 1972; Olafson and Thompson, 1974).

CHAPTER III

MATERIALS AND METHODS

Organisms

The organisms used in this study are listed in Table 1 with their strain designations, sources, and relevant citations (if any). The primary study organism, Arthrobacter marinus, was originally isolated from intertidal waters of a north-eastern U.S. bay, on the basis of resistance to Ni.

Stock cultures of most strains were maintained on slants of 2216E modified agar (see below). Those cultures not showing good growth on this medium were cultured on Trypticase Soy Agar (BBL) or on 424 agar for hydroxamate-requiring Arthrobacter species. Stocks of Arthrobacter marinus LAB and its conspecifics were maintained by frozen storage (Chesbro, personal communication). Cells were harvested by centrifugation from broth culture and resuspended in 10 % dimethyl sulfoxide (DMSO). Cryoprotected cells were frozen at -92°C in a Revco Ultralow freezer. At three to six month intervals a set of cultures was thawed, an aliquant transferred to 2216E modified broth, grown out, and transferred to agar slants. Agar slant cultures were grown at 25°C until good growth was apparent, then stored at 4°C.

Media

The majority of experiments were performed in artificial seawater media. Artificial seawater was prepared using the

Table 1. Bacterial strains and sources

Species	Strain	Source	Citation
Arthrobacter marinus	Lab	UNH CC[1]	Cobet, Wirsen, and Jones (1970)
Arthrobacter marinus	R1	JEL[2]	Wirsen (1966)
Arthrobacter marinus	R2	JEL[3]	Wirsen (1966)
Arthrobacter marinus	ATCC 25374	ATCC[4]	Cobet, Wirsen, and Jones (1970)
Arthrobacter globiformis	8010	ATCC	
Arthrobacter crystallopoites	15481	ATCC	
Arthrobacter terregens	13345	ATCC	
Arthrobacter simplex	6946	ATCC	
Alcaligenes cupidus	27124	ATCC	
A. faecalis	8570	ATCC	
A. paradoxus	17713	ATCC	
Rhizobium trifolii	14480	ATCC	
R. leguminosarum	10004	ATCC	
Agrobacterium tumefaciens	4720	ATCC	
Agrobacterium luteum	25657	ATCC	

[1]University of New Hampshire Culture Collection

[2]Isolated from original stock slant of Wirsen (JEL cold room)

[3]Isolated from original stock slant of Wirsen (containing 4×10^{-4}M Ni, from JEL cold room)

[4]American Type Culture Collection

Table 1 Continued

Species	Strain	Source	Citation
Achromobacter aquamarinus	14406	ATCC	
Pseudomonas marina	27129	ATCC	Baumann *et al.* (1972)
P. putida (*P. cuprodurans*)	29735	ATCC	Passman and Jones (in press)
P. iodinum		UNH CC	
P. putida		UNH CC	
P. sp 130	130	NCMB[5]	
Alteromonas haloplanktis	B-16	UNH CC	
Bacillus subtilis var. *niger*	BG	NCI[6]	
Brevibacter linens		UNH CC	
Kurthia zopfii		UNH CC	
Escherichia coli	CSH26	UNH CC	
E. coli	4BFW26	UNH CC	
E. coli	W1A2	UNH CC	
E. coli	HFR-C	UNH CC	
Salmonella gallinarum		UNH CC	

[5]National Collection of Marine Bacteria, Britain

[6]National Cancer Institute, M. Barbeito, Bethesda, MD.

formula of Lyman and Fleming (1940) (designated L&FASW) or the formula of Kester et al. (1967) (designated KASW). KASW, while more tedious to prepare, gave superior results with respect to final pH and the formation of precipitates during autoclaving. For a limited number of experiments, the commercial artificial seawater "Seven-Seas Marine Mix" (Utility Chemical Company) was prepared according to manufacturer's instructions. Artificial seawater was diluted to 75 % of full strength (26.25 g/kg salinity) before use.

To avoid uncontrolled variations of media or glassware due to water quality, doubly distilled, deionized water was used for most procedures. High purity water was prepared by redistilling centrally supplied single distilled (tin plated still) water in an all-glass Corning AG-11 automatic still and feeding the output to a Millipore Milli-Q water polishing system. The Milli-Q system provided resin deionization, activated carbon removal of organic compounds, and final 0.2-μm filtration. This system provided point of use water quality of 1.5 to 1.8 X 10^7 ohm/cm resistivity (termed QH_2O).

Complex media were prepared as modifications of the 2216 formulation of ZoBell (1946). 2216E modified (2216E MOD) was prepared as based on Oppenheimer and ZoBell (1952): 1.0 g peptone (Fisher Biocert), 1.0 g yeast extract (BBL), 1000 ml 75 % ASW. For a solid medium, 15 g agar (Difco) per liter was added and dissolved by heating prior to autoclaving. A medium having one-half of the nutrient concentrations of the above medium was designated 2216E LN (low nutrient). For some purposes the commercial medium Difco Marine Agar was prepared

according to the manufacturer's instructions. For the cultivation of some of the terrestrial bacteria studied, BBL Trypticase-Soy Broth (TSB) with or without 15 g/liter agar (TSA) was used. For the cultivation of hydroxamic acid-requiring strains of Arthrobacter (Arthrobacter flavescens, Arthrobacter terregenes, Arthrobacter simplex) the ATCC recommended medium (424) was used: 10 g peptone, 10 g yeast extract, 2 g K_2HPO_4, and 40 µg Desferal (Ciba) per liter QH_2O. To obtain good growth of the Rhizobium cultures used, it was necessary to use the ATCC recommended 111 medium: 10 g yeast extract, 10 g mannitol, 200 ml soil extract, 800 ml QH_2O. Soil extract was prepared by adding 805 g commercial potting soil (Swiss Farms "sterilized scientifically prepared soil for African violets", Swiss Farms, Inc.) to 2.09 g Na_2CO_3 in 2091 ml QH_2O, autoclaving 1 h, allowing to settle, and the supernatent filtered through Whatman # 1 (Whatman, Inc.) filter paper prior to use.

A defined medium (designated M-9), used for the majority of experiments contained: NH_4Cl 1.0 g, K_2HPO_4 0.25 g, anhydrous D-glucose (dextrose) 5.0 g, 1000 ml 75 % ASW. The medium was designated unbuffered M-9 (UB-M9). The medium was also used in a buffered form. The buffers tris-(hydroxymethyl)-aminomethane (Tris), N-2-hydroxyethyl-piperizine-N'-2-ethanesulfonic acid (HEPES), piperizine-N,N'-bis(2-ethane-sulfonic acid) (PIPES) (Calbiochem), were used in 0.0413 M concentration (5.00, 9.84, 14.13 g/liter, respectively) giving media designated TRIS-M9, HEPES-M9, PIPES-M9.

In order to minimize the formation of calcium and/or magnesium phosphate precipitates and the caramelization of the sugar, all of the M-9 media were made by a three step procedure. Firstly, the seawater, NH_4Cl, and buffer (if any) were mixed, adjusted to a pH of 7.5 to 7.8, dispensed, and autoclaved. Secondly, the cooled, autoclaved medium was adjusted to pH 7.4 by titrating one or more samples with sterile 0.05 N HCl, and adding the required volume of sterile acid to each flask. Finally, sterile stocks of phosphate (as 1.0 ml/100 ml of a 0.025 g/ml K_2HPO_4), and glucose (as 1.0 ml/100 of a 0.5 g/ml anhydrous D-glucose solution) were added aseptically to the medium. Care was exercised to insure that the contents of each flask of medium were mixed thoroughly both after the addition of acid and immediately following the addition of phosphate to avoid the formation of a stable phosphate precipitate. Even with these precautions, there was a transient formation of precipitate upon the addition of phosphate. However, this redissolved when the flask was promptly mixed.

For most experiments, medium was used as broth, dispensed as 100-ml aliquots in 250-ml Erlenmeyer flasks. Flasks were closed with Kim-Wipe (Kimberly-Clark) enclosed wads of Fisher non-absorbent cotton, or open-cell polyurethane foam plugs (VWR Scientific). When used, nickel was added as the appropriate aliquant of 0.1 or 0.001 M $NiCl_2$ prior to autoclaving.

Media were sterilized by autoclave for most experiments. For a small number of experiments sterilization by membrane filtration was used. A variety of Millipore filtration

apparatus was used, including 47-mm all-glass filter holders, 47-mm "Steri-fil" plastic filter units, and a 600-ml pressure filter reservoir with 47-mm "Swinnex" inline filter holder. To insure filtration effectiveness, 0.2-µm filters were used for sterile filtration.

The metal content of the membrane filter materials was determined to insure that membrane filtration would not add to the trace metal burden of media. Samples of both Millipore HA cellulosic filters and Nuclepore polycarbonate filters were wet-ashed and their metal content determined (see metal analysis below). Ni, Pb, Co, Mn, and Cd were below the detection limit for both filter types. The Millipore filters contained 0.90 µg/filter (47-mm diameter) Cu, 0.83 µg/filter Fe, and 0.29 µg/filter Zn. The Nuclepore filters were much lower in metals, 0.01 µg/filter Cu, 0.04 µg/filter Fe, and 0.008 µg/filter Zn. For both filter types the metal content was low enough to be of no routine concern. For the most demanding applications, the Nuclepore filters were used.

The trace metal content of the media used, particularly of Ni, was quantitated (see metal analysis techniques below) to allow determination of background metal effects. Table 2 presents the content for four metals of artificial seawater, three media, and a sample of natural seawater. The trace metal content of KASW is slightly less than, but comparable to, the natural seawater sample, with the exception of undetectable Ni in the KASW. Comparison of the values with the mean seawater estimates of Goldberg (Table 2) shows both Great Bay seawater and KASW to be lower by factors of 2 to 5 or more in the four metals.

Table 2. Trace element content of artificial seawater (KASW) media (UB-M9, 2216E LN, 2216E MOD) and natural seawater measured by APCD-MIBK extraction and atomic absorption spectroscopy. First value is µg/liter, parenthetic value is molarity.

Sample	Ni	Co	Cu	Zn
KASW[1]	0.0 (-)	0.0 (-)	0.84 (1.3×10^{-8})	1.1 (1.7×10^{-8})
UB-M9	0.51 (8.7×10^{-9})	0.0 (-)	1.0 (1.6×10^{-8})	5.3 (8.1×10^{-8})
2216E MOD	8.8 (1.5×10^{-7})	2.3 (3.9×10^{-8})	39 (6.1×10^{-7})	82 (1.2×10^{-6})
Great Bay[2] Water	1.0 (1.7×10^{-8})	0.0 (-)	1.6 (2.5×10^{-8})	2.6 (4.0×10^{-8})
Mean Seawater[3] Estimate	2.0 (3.4×10^{-8})	0.1 (1.7×10^{-9})	3.0 (4.7×10^{-8})	10 (1.5×10^{-7})
Detection Limit	c.0.03 (5.1×10^{-10})	c. 0.05 (8.5×10^{-10})	0.05 (7.9×10^{-10})	c. 0.01 (1.5×10^{-9})

[1]Mean of 4 samples.

[2]Mean of 2 samples.

[3]Goldberg (1965).

Addition of the nutrients giving UB-M9 medium raised the metal concentration to levels comparable to Great Bay water but 2 to 4-fold lower than mean seawater. By contrast, addition of 1.0 g each of peptone and yeast extract (2216E MOD) raised metal concentrations by factors of forty-fold or more compared to KASW. Attempts to analyze the metal content of 2216E LN were unsuccessful, giving contradictory results. The metal content of peptone and yeast extract was analyzed independently by wet ashing. Those results are presented in Table 3, with the metal content of 2216E LN and MOD media calculated from the nutrient concentrations and the analysis of KASW. The calculated metal content was greater than was measured (Table 2) with the exception of Cu in 2216E MOD. The metal contents of the two nutrients were comparable with those previously reported for tryptone and yeast extract (Jones, Royle, and Murray, 1976). The differences between the metal ion content of 2216E-type media was measured by solvent extraction and as calculated by independent analysis of the constituents may reflect trace metal chelation by the organic constituents of the medium which made the metals inaccessible to the extracting chelator. The calculated 2216E medium metal contents were considered more valid.

The metal content of UB-M9 medium also was analyzed by a separate technique. UB-M9 medium (4.5 liter) was extracted by Chelex-100. The results of atomic absorption analysis of the eluate is shown in Table 4 with the solvent extraction results given for comparison. While individual metals show differences, the results are roughly comparable.

Table 3. Atomic absorption spectroscopy analysis of the metal content (µg/g) of peptone (Fisher Biocert) and yeast extract (BBL), and the calculated 2216E LN (0.5 g peptone, 0.5 g yeast extract) and 2216E MOD (1.0 g peptone, 1.0 g yeast extract) content.

Metal	Peptone	Yeast Extract	Calculated 2216E LN		Calculated 2216E MOD	
			µg/liter	molarity	µg/liter	molarity
	µg/g	µg/g				
Ni	8.4	6.5	7.5[1]	1.3×10^{-7}	15.0[1]	2.5×10^{-7}
Fe	45	30	37	6.7×10^{-7}	75	1.3×10^{-6}
Zn	55	52	55[1]	8.2×10^{-7}	108[1]	1.6×10^{-6}
Co	3.8	5.5	4.7[1]	7.9×10^{-8}	9.3[1]	1.6×10^{-7}
Cu	3.6	4.5	4.9[1]	6.4×10^{-8}	8.9[1]	1.3×10^{-7}

[1]Includes metal content measured of KASW (Table 2).

Table 4. Analysis of UB-M9[a] by Chelex 100 extraction-elution[b].

Metal	Concentration Chelex-100 µg/liter	Concentration[c] APCD-MIBK µg/liter
Ni	2.7	0.51
Cu	2.2	1.0
Pb	0.60	
Co	0.0	0.0
Fe	2.1	
Zn	2.7	5.3
Mn	0.36	
Cd	0.50	

a. 4.5 liters of UB-M9 passed through column

b. resin bed eluted into total volume of 10.0 ml 1 N HNO_3

c. data from Table 3 included for comparison

When the background Ni concentration of UB-M9 medium was needed, the 2.7 µg/liter value was used as the Chelex-100 technique was considered to be more direct than the solvent extraction technique.

Glassware

All glassware used for the preparation of media, the culturing and manipulation of organisms, and the analysis of metals and metal complexation, was cleaned scrupulously to reduce extraneous metal or organic contamination. Immediately after use, all glassware was soaked in a disinfectant (Roccal II, National Laboratories). At intervals, glassware was removed for washing by a mechanical dishwasher (Better Built) using high temperature detergent with a single distilled water rinse. Machine-washed glassware was stored in dust-free cabinets for direct use, or for critical purposes, was acid-washed. Acid washing entailed a 24 h (or longer) soak in concentrated nitric acid, draining of excess acid in a drip tank for 24 h (or longer), by a single QH_2O rinse, and a 24 h (or longer) soak in QH_2O. Next the glassware was rinsed extensively in running QH_2O. Typically, 5 - 10 rinses were required to remove all traces of acid, as shown by pH testing of the rinse water. Finally, the items were dried in as dust-free an environment as possible (a laminar flow clean bench when available, Model CVF42-36B1, The Baker Co., Inc.). Finished glassware was covered with polyethylene film (Saran Wrap) and stored in dust-free cabinets.

Certain items were harmed by the concentrated nitric acid soak. These included polycarbonate plastic items, such as centrifuge bottles, Millipore "Steri-fil" filtration units, and the barrel of the 600-ml pressure-filter reservoir. Such items as filter O-rings were also attacked by nitric acid. If necessary, such items were acid-washed by brief soaking in 1 N HCl followed by rinsing as above.

Measurement of Growth

In routine experiments, growth was measured as optical density (OD) at a wavelength of 420 nm (OD_{420}), using matched 1-, 2-, or 5-cm optical glass cuvettes in a Zeiss PMQ-II spectrophotometer blanked against QH_2O. All readings were normalized to 1-cm effective pathlength when longer cuvettes were used. Routinely, samples having 1-cm OD values of 1.0 or greater were diluted with sterile seawater and reread; the diluted OD was multiplied by the dilution factor to give a corrected OD.

Though the measurement of bacterial growth by OD is perhaps the most routinely used technique by bacteriologists (Koch, 1968), there are a number of factors affecting the measurement which are comprehended poorly by most users (Koch and Crandall, 1968). Due to the nature of bacterial light scattering and the differing light source-cuvette-detector geometry of commercial spectrophotometers, the measured OD of a suspension of bacteria will not be identical on different instruments (Koch and Crandall, 1968). An OD 1.04 cell suspension on the PMQ-II spectrophotometer measured 0.620 on a

Spectronic-20 (Bausch and Lomb) spectrophotometer. The detector geometry of the PMQ-II spectrophotometer most closely approximates that of an ideal turbidimeter (Koch, 1970).

Further, at OD values greater than 0.5 to 1.0 (dependent on instrument) a significant deviation of OD curves from linearity occurs (Koch and Crandall, 1968; Lawrence and Maier, 1977). The point at which deviation from linearity occurred for A. marinus in the PMQ-II spectrophotometer was determined. A singly washed suspension of UB-M9 grown cells (OD 5.08, by dilution) was diluted to give 25 suspensions of dilution factor 1.00 to 0.030 (calculated OD 5.08 to 0.152) and the OD of each suspension measured (Table 5). At observed OD values greater than 0.51, there was a negative deviation. Thus, the observed OD was less than its true value. The magnitude of the deviation increased with increasing OD, becoming significant above OD 1.0. When plotted, the deviation appeared to be exponential and the $-\log_{10}$ of the deviation plotted against the observed OD approximated a linear function. For routine purposes, samples were diluted for OD measurement when the OD of the undiluted culture was greater than 1.0. For the most precise measurements, dilution above OD 0.6 was preferable.

Five other growth parameters were evaluated as alternatives to the turbidimetric technique for quantitation of growth. Viable count was determined by appropriate dilution of the culture in sterile 75 % ASW and replicate spread plating of 0.1-ml aliquants on 2216E MOD agar. Plates were counted after 10 to 14 day incubation at 20°C. Counts were expressed as colony forming units (CFU) per ml.

Table 5. Effect of dilution on the OD of *A. marinus* grown in UB-M9, washed once, and resuspended in 75 % KASW.

Dilution factor	OD Expected[1]	OD Observed	Obs. - Exp.[2]
1.00	5.08	>2	-
0.90	4.57	>2	-
0.80	4.06	>2	-
0.70	3.56	>2	-
0.60	3.05	2.0	-1.05
0.50	2.54	1.9	-0.64
0.45	2.27	1.8	-0.47
0.40	2.03	1.68	-0.35
0.35	1.78	1.46	-0.32
0.30	1.52	1.30	-0.22
0.25	1.27	1.11	-0.16
0.225	1.13	1.03	-0.10
0.20	1.01	0.93	-0.080
0.175	0.889	0.83	-0.060
0.15	0.762	0.72	-0.040
0.10	0.508	0.506	-0.002
0.090	0.457	0.464	0.007
0.080	0.406	0.407	0.001
0.070	0.356	0.350	-0.006
0.060	0.305	0.308	0.003
0.050	0.254	0.255	0.001

Table 5 continued.

Dilution factor	OD Expected	OD Observed	Obs. - Exp.
0.045	0.227	0.245	0.018
0.040	0.203	0.197	0.006
0.035	0.178	0.189	0.011
0.030	0.152	0.155	0.005

1. calculated from OD 5.08 original suspension, measured by dilution
2. OD observed minus OD expected

Total count was determined by a calibrated microslide technique. Ten µl was dispensed onto a standard microscope slide using an Oxford Precision pipettor. The droplet was covered with a standard 22-mm # 1 cover slip encompassing an area of 4.84 cm^2. Dividing the volume (1 X $10^{-2} cm^2$) by the area gave a calculated pathlength of 20.7 µm. The oil immersion objective of the microscope covered a field of 122 µm diameter, or 1.17 X 10^4 μm^2 and viewed a volume of 2.42 X 10^{-4}µl. Thus, to see one cell per field required 4.13 X 10^6 cells/ml. A sweep of the coverslip from edge to edge covered a rectangular field 122 µm X 22 mm, a volume of 5.55 X 10^{-2}µl. This corresponded to 1.8 X 10^4 cells/ml for each cell observed.

Cell mass, as µg dry weight/ml, was measured by filtering an appropriate aliquant through a tared 25-mm 0.4-µm Nuclepore polycarbonate filter. Statistical analysis of filters which were washed with QH_2O or 75 % KASW and fixed or unfixed by 5 % formaldehyde showed no significant variation due to wash or fix. Therefore, results using different wash procedures were pooled for analysis. Filters were dried at 60°C, reweighed, and a dry cell mass per ml calculated. Weighings were performed on a Cahn Model # 4400 Electrobalance.

Following weighing, the filters were used to determine a fourth parameter, cell protein. The technique used was modified extensively from that of Lowry et al. (1951), with modifications suggested by Toennies and Feng (1965) and Chesbro (personal communication). The following reagents were prepared: A. 25 w/v Na_2CO_3 in 0.1 N NaOH; B-1. 5 % w/v $CuSO_4 \cdot 5H_2O$ (3.2 % anhydrous $CuSO_4$) in QH_2O; B-2. 10 % w/v potassium

sodium tartrate in QH_2O; B. 1 volume B-2 + 8 volumes QH_2O + 1 volume B-1; C. 50 volumes A + 1 volume B; D. 0.5 N Folin phenol reagent (Fisher Folin-Ciocalteu 2 N reagent diluted 1:4). The procedure was as follows: 1) to each filter in a 16 X 150-mm test tube was added 5 ml reagent A followed by 1 h incubation at 25°C, mixing with a vortex mixer at 15 min intervals (this hydrolyzed the cell protein); 2) to 1 ml of the solution A hydrolyzed cells, 4 ml solution C was added followed by 30 min incubation at 25°C; 3) 1 ml solution D was added to each tube, vortexed immediately, and incubated 30 min at 25°C; 4) the optical density at 500 or 750 nm was read comparing sample optical density to that of standards made by diluting a 5-mg/ml solution of bovine serum fraction V (Calbiochem) appropriately in solution A and treated identically with the samples. For the analysis of cells on filters, a standard curve of 0 to 50 µg/ml protein was used. However, the technique gave linear results to at least 500 µg/ml protein. The 750 nm wavelength gave slightly greater sensitivity whereas 500 nm produced superior linearity. The technique was modified readily to analyze samples other than dried cells on filters. Other samples were adapted by hydrolyzing in solution A and using the solution as the sample in step 2. However, samples in the growth media must be removed from their solution before analysis as glucose, TRIS, K^+, Mg^{++} interfere with the technique.

From OD_{420} growth data, three parameters descriptive of growth were derived. These were doubling time (DT), maximum OD_{420} (OD_{MAX}), and lag time (Lag). The OD_{MAX} was determined

by examination of the data. Lag (h) was determined by examination of the data and growth curves. The lag time was the period of time after inoculation prior to the initiation of logarithmic growth. When the start of growth clearly had occurred between two observation times the lag time was interpolated between the two times. When doubt existed as to the actual lag time, it was indicated as circa a given time (i.e. c. 95 h). DT was determined from paired time and OD_{420} values which when possible fell within the log phase of growth. Doubling time was calculated by the formula derived from the discussion of exponential growth of Stanier, Doudoroff, and Adelberg (1970):

$$N_t/N_o=2^{k\Delta t}$$

$$\log_2(N_t/N_o)=k\Delta t$$

$$k=(\log_2(N_t/N_o))/\Delta t; \quad DT=1/k$$

$$DT=\Delta t/\log_2(N_t/N_o): \quad \log_2 x=\log_{10} x/0.301$$

$$DT=0.301\Delta t/\log_{10}(N_t/N_o)$$

The final transformation was used to prepare programs both for the Monroe 324 Programable Calculator, and in the BASIC programming language for the DEC-10 (Digital Equipment Corporation) time-sharing computer. In the later stages of M9 growth experiments which frequently extended 10 to 30 days, it was necessary to sample experimental flasks at intervals of 1, 2, or more days in order to avoid using up the flask volume. Occasionally, in between sampling periods the entire growth phase passed. In that case, the DT was determined from the OD increase between the two observations but such a value would

be larger than the actual DT of the culture during growth. Thus differences between DT values must be viewed with caution.

Inocula and Growth Conditions

For all growth experiments the first step was the inoculation of a 100 ml 2216E LN broth from a stock slant. At 18 to 24 h incubation this culture was in late log or early stationary phase (OD_{420} 1.5 - 1.8) and was used directly to inoculate the experiment if 2216E LN medium was used. For experiments in M9 medium the 2216E LN starter was used to inoculate (1 ml/100 ml of medium) a broth culture of M9 medium. After 18 to 24 h, this culture was in late log phase (OD_{420} 1 - 3) and was used to inoculate the experimental flasks.

Dependent on the experiment, the broth cultures were used directly or washed prior to use. Washing was by centrifugation of the cells at 10,000 X g in a Sorvall RC-2B refrigerated centrifuge (4°C) and resuspension of the cell pellet into an equal volume of sterile 75 % ASW. Inocula were standardized via an optical density technique in order to give readily comparable results. The OD_{420} of the inoculum culture or washed cell suspension was measured and the value normalized to an OD of 1.00. An inoculum of 1.0 ml of an OD 1.0 cell suspension per 100 ml was termed a 1.0 EQ inoculum, 0.1 ml of OD 1.0 was 0.1 EQ, etc. Thus, for a 1.0 EQ inoculum from an OD 1.4 starter culture, 0.714 ml per 100 ml was used.

Unless otherwise specified, experiments were incubated at 25°C in a New Brunswick Scientific Psychrotherm Model G-26 gyrotatory incubator shaker. Broth cultures were shaken at

200 rpm. Static cultures were incubated on a shelf in the same unit.

Optical Microscopy

The primary optical microscope utilized was a Zeiss WL Research Microscope equipped with fluorite phase contrast objectives and a VZ phase condenser. This equipment allowed the use of conventional brightfield, phase contrast, and dark-field microscopy. Photomicrographs were taken using several different cameras. The primary camera used was a Reichert Remica III focusing unit with a 35-mm camera back. Kodak Tri-X film was used with this camera with exposures empirically determined for each illumination technique and magnification. A Leitz 4 X 5-in-view camera using either Kodak 4 X 5-in Plus-X cut film in Graphloc holders or Polaroid type 55 PN 4 X 5-in sheet film in a Polaroid # 500 film back was used for some experiments. For photomicrography using color film, an Olympus Model PM-7 camera with C-35 film transport and EMM-6 color compensating exposure meter was used on the Zeiss microscope. Kodak tungsten balanced Ektachrome EHB 35-mm film processed to a speed of 320 ASA was used. In order to get a proper color balance in the resulting transparencies, a lighter blue LB 45 filter was used rather than the LB 200 filter indicated by the exposure meter color compensating curcuit.

In addition to the standard 10 X compensating oculars, the microscope was equipped with a 12.5 X ocular micrometer. This was calibrated against a Zeiss stage micrometer for precise measurement of cells. The stage micrometer was also used to determine the field of the individual objectives.

Electron Microscopy

Samples for electron microscopy were prepared as whole cell mounts on carbon-stabilized formvar coated 300 mesh copper electron microscope grids. Following poor results using platinum-carbon shadow cast and 1.0 % potassium phosphotungstate (pH 7.4), the technique of Allen and Baumann (1971), using 1 % uranyl acetate at pH 4.2 was used with success. Prepared grids were examined in a Phillips Model 200 transmission electron microscope at magnifications from 1,000 to 50,000 X. Electron micrographs were taken on glass plates or cut film.

Radioisotope Techniques

Four radionuclides were used. ^{3}H (as methyl-^{3}H-thymidine), ^{14}C (as uniformly labeled glucose), ^{32}P (as K_2HPO_4), and ^{63}Ni (as $NiCl_2$) were all obtained from New England Nuclear. The isotopes were soft to moderate β-particle emitters, and were counted using liquid scintillation counting (LSC) techniques. A Packard Tri-Carb Model 3320 was used for counting. For the ^{3}H, ^{14}C, and ^{32}P isotopes standard Packard counting parameters were used.

For the nonstandard Ni isotope, gain was optimal at an instrument setting of 35 %. Corrected absolute activity of samples (decays per min, DPM) was determined by the automatic external standardization technique (AES). Efficiency versus AES count was determined by adding a known amount of ^{63}Ni (1×10^{5} DPM, 4.5×10^{-2} micro Curies (μ Ci)) to 15-ml volumes of scintillation fluid quenched by graded amounts of

water, cells, or Millipore filter material and counted. From the ratio of counts per min (CPM) to DPM added the efficiency was calculated. The graph of efficiency versus log AES count was a linear function in the range of 40 - 50 % efficiency in which most counts fell. From this observation, an automatic program for the Monroe Model 324 Programmable Calculator allowed the automatic calculation of DPM values from input raw counts. Due to an irregular quench effect at low amounts of water in the scintillation fluid, it was adopted as standard procedure to add samples as 1 ml aqueous solutions, or if the samples were dried or organic solutions, 1 ml of QH_2O was added per vial. For liquid scintillation counting procedures, 15 ml per vial quantities of New England Nuclear Aquasol Universal LSC Cocktail was used.

Measurement of pH

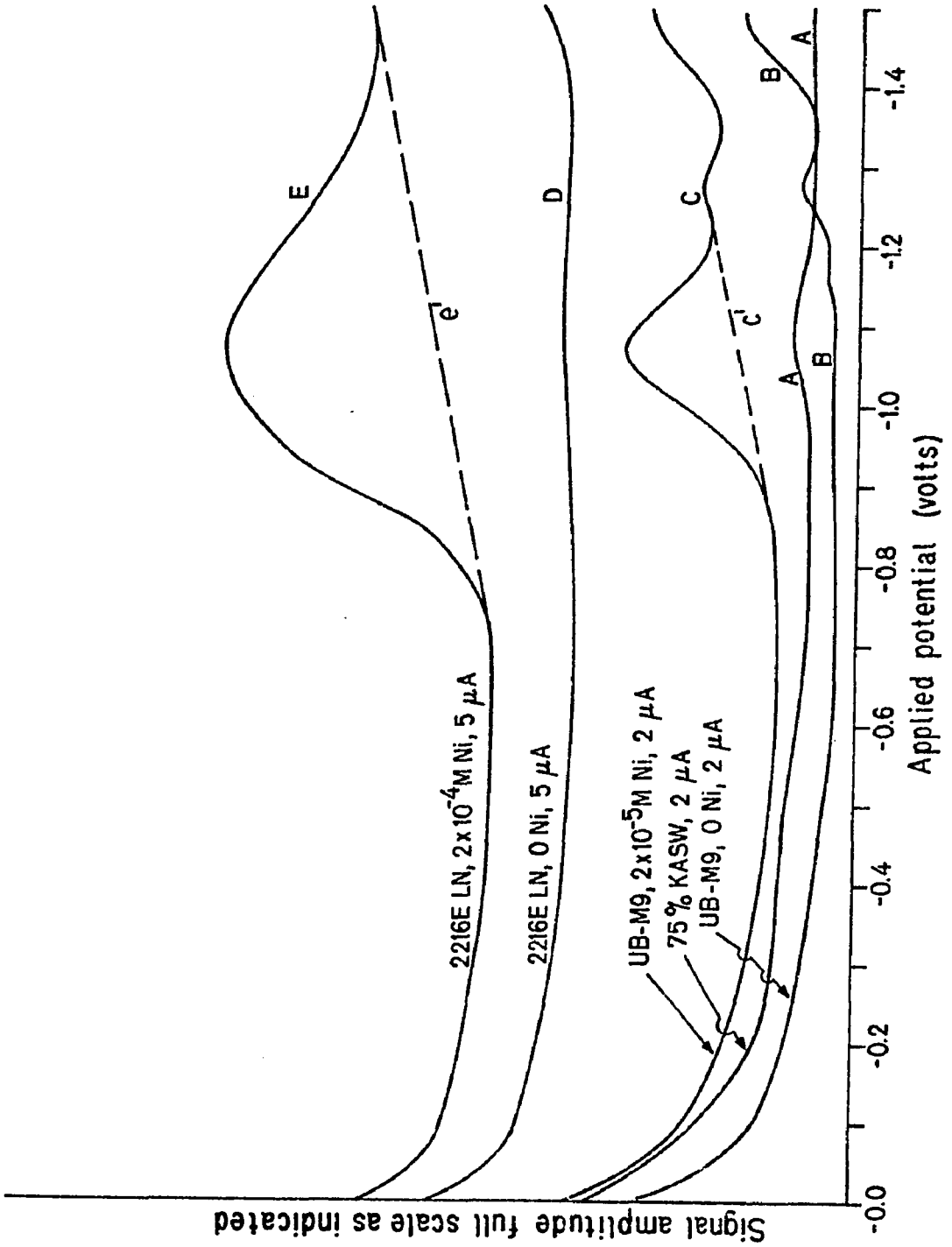
A Corning Model 12 pH meter was used to measure pH; expanded scale gave a full scale reading of 1.0 pH unit. Probes used were Corning Model 476050 or Markson (Markson Science, Inc.) Model JM-1885 "PolyMark" semi-micro sized combination electrodes. These were of a size which allowed pH to be measured on a 2.75 ml sample in a standard 1-cm spectrophotometer cuvette. The pH meter was standardized against commercial (Fisher, Corning, and Scientific Products) buffers at pH 4.01 and 7.00 which allowed accurate temperature compensation.

Differential Electrochemical Analysis

For a variety of experiments electrochemical analyses were performed with a Princeton Applied Research (PAR) Model 174A Polarographic Analyzer interfaced to a Model 315 Automated Electroanalysis Controller and a Houston Instruments Omnigraphic Model 2000 Flat Bed XY Axis Recorder. Dependent on the electrochemical procedure utilized, either a PAR Model 172 Dropping Mercury Electrode or a 9323 Hanging Drop Mercury Electrode were used. For some of the experiments the instrument was operated by Mr. L. J. Spencer who also contributed to the analysis of electrochemical data. While the instrument was capable of a variety of analytical modes, the two used were differential pulse anodic stripping voltammetry (DPASV) and differential pulse polarography (DPP). The former technique has superior sensitivity but is operationally more difficult. In the systems studied, the enhanced sensitivity was unnecessary. Therefore, the DPP technique was used exclusively. This technique produced an output plot (Fig. 1) of voltage versus current. A metal ion produced a peak at a voltage equal to its reduction potential in the electrolyte used and a height (current) proportional to its concentration. The reduction potential of the peak was termed the $E_{1/2}$ and the height (in units of current, typically μA) was termed i.

Frequently the polarogram, rather than exhibiting simple gaussian peaks on a straight baseline, showed a pattern of multiple overlapping peaks on a rising or falling baseline. The resolution and quantitation of individual peaks under these conditions presented a difficult and only partially

Fig. 1. Representative differential pulse polarograms. Scan range: 0.0 to -1.5 v, drop time: 2 sec, scan rate: 5 mv/sec, sensitivity (full scale deflection) as given.


Signal amplitude full scale as indicated
Applied potential (volts)
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
2216E LN, 2x10⁻⁴M Ni, 5 μA
2216E LN, 0 Ni, 5 μA
UB-M9, 2x10⁻⁵M Ni, 2 μA
75% KASW, 2 μA
UB-M9, 0 Ni, 2 μA
E
e
D
C
c
B
A
A
B

solvable problem. Two principal techniques of polarogram analysis were used. In the first, the peak was quantitated by peak height from a baseline. This was done (Fig. 1) by drawing a line tangent to the bases of the peak and measuring from it. The placement of the baseline was critical to quantitation. In the case of complex waveforms or of a small peak superimposed on the rising or falling edge of a much larger peak, it was subject to uncertainty. The second technique was by peak area. Area was measured directly by cutting out the peak and weighing the graph paper, or by use of a planimeter; or by the use of an analog computer, a Dupont Model 310 Curve Resolver. This device allowed both the deconvolution of complex waveforms into individual gaussian peaks, and the measurement of the area of the resulting simple peak. However, curves on a steeply sloping baseline, or small peaks superimposed on a larger, off-scale peak may be influenced by small variations in the approximation of the large peak.

Polarograms were conducted generally at 0.0 to -1.5 v scan ranges at a drop time of 2 sec per drop, a scan rate of 2 mv per sec, and sensitivies of from 0.5 to 5 μA full scale. For comparison, the drop time and scan rate had to be identical. To compare scans made at differing sensitivities a scale factor was applied though sensitivity changes of a factor of 10 or greater made quantitative intercomparison risky. For many experiments, the intercomparison of curves was facilitated by a control sample tested immediately before or after the test sample.

Ultrafiltration

Ultrafiltration for the detection of Ni bound by moderate molecular weight organic compounds was performed in a Nuclepore Radial Flow Cell 90-mm filter holder in a recirculating mode pumped by a Cole Parmer Masterflex 7545-10 Variable Speed Drive with 7016 pumphead. Pressure across the filter membrane was maintained by restricting the output flow with a regulating valve. The ultrafiltration membrane was an Amicon UM-05 having 100 nm nominal porosity giving a c. 500 dalton molecular weight cut-off. One hundred-ml volumes of ^{63}Ni labeled medium filtered through 0.4-µm Nuclepore or Millipore membranes to remove cells or precipitated particles were recirculated through the filter holder. Samples of both permeate and retentate were collected at intervals up to the permeate volume of 50 ml at which point the remaining retentate volume was equal to the dead volume of the filtration system and the pump began to draw air. One-ml samples of the permeate and retentate were counted using radioisotope techniques and the relative retention or permeation determined from the corrected DPM values. An initial experiment was performed using a solution of 0.1 % bovine serum fraction V in QH_2O. Permeate and retentate were sampled at volumes of 0, 10, 25, and 50 ml permeate volume and the high molecular weight protein quantitated by OD at 280 nm. From the OD_{280} values the percentage retention was calculated by the formula given in Amicon literature:

$$R\ \% = (\ln(C_f/C_o))/\ln(V_o/V_f) \times 100$$

where ln indicated natural (base e) logarithm, C_f and C_o

were the solute concentration in the retentate at the end and beginning, respectively. V_f and V_o were the retentate volume at the end and beginning, respectively.

Dialysis

Binding of Ni to high molecular weight organic compounds was assayed by determining the ability of Ni as ^{63}Ni to permeate a dialysis membrane. Spectrapore 3 (Spectrum Medical Industries, Inc.) dialysis tubing (3500 dalton molecular weight) was prepared according to manufacturer's instructions. Ten-ml aliquants of filtered growth medium (UB-M9) labeled with ^{63}Ni were dialyzed against an equal volume of QH_2O at 4°C for 24 h. The ^{63}Ni content of 1.0-ml aliquants of the retentate and dialysate were measured by LSC.

Gel Permeation Chromatography

The technique of gel permeation chromatography (Anonymous, 1971) was applied to the detection of Ni-binding by organic compounds in culture supernatants. A gel permeation column was prepared using Bio-Rad P-2 200-400 mesh resin (Bio-Rad Laboratories). This resin had an exclusion limit of 1800 daltons. A 15 X 250 mm Chromatoflow Series B column (Pierce Chemical Co.) was packed to a depth of 213 mm using the resin manufaturer's recommended technique. The void volume of the column was determined using Blue Dextran 200 (Pharmacia), an extremely high molecular weight cross-linked polysaccharide which was excluded from the interior of gel particles and migrated in the void volume of the column. The marker was eluted from the column (using 75 % KASW as the eluting buffer)

at a volume of 13.2 ml. Flow through the column using a Mariotte flask technique (Bio-Rad Laboratories) maintained a constant hydrostatic head of 68 cm which gave a flow rate of 2.2 ml/min (6 min/void volume).

The occurrence of Ni-binding organic compounds in culture supernatants was assayed by loading 2-ml samples of the filtered culture onto the column and eluting with 75 % KASW and sampling at periods of 1/3 void volume (2 min) using a semi-automatic fraction collector (a test tube rack and stop watch). One-ml samples were counted using standard LSC techniques and the DPM values plotted.

Chelex-100 Resin Chromatography

The technique of Stolzberg and Rosin (1977) used chromatography past a short plug of the high-avidity chelating ion exchange resin, Chelex-100 (Bio-Rad Laboratories) to estimate the content of copper-complexing organic matter in phytoplankton media and natural water samples. The techniques as modified here utilized a short (15 X 30 mm) column of 100-200 mesh Na-form resin (batch # 12667) equilibrated to pH 7.9 (initial pH c. 10) with 1 N redistilled HCl (the pH equilibration of the resin was very slow, adjustment requiring 12 h). Initial experiments were conducted evaluating the performance of a second chelating chromatographic resin, 8-hydroxy-quinoline linked 20-80 mesh Corning Controlled Pore Glass (8HQ-CPG, Corning Biomaterials) (Sugawara, Weetal, and Schucker, 1974). This material was used as received from the supplier (Pierce Chemical Company).

The metal binding capacity of each resin was determined by passing 1000 ml of 1 X 10^{-4}M $NiCl_2$ in 75 % KASW through each column, labeled with 1.2 nCi/ml (2664 DPM/ml) of ^{63}Ni. The flow rate was c. 5 ml/min through each column. The input solution and the effluent at 100-ml effluent volume intervals were sampled and counted by LSC. Following passage of the sample, the two columns were eluted with 10-ml volumes of 1 N HNO_3 and washed with three 25-ml volumes of QH_2O counted to determine the column extraction and recovery efficiencies. Chelex-100 resin remained highly acid after exhaustive washings. Thus, rather than regenerating the column in situ, the column was cleaned and refilled with fresh resin after each experimental series. Based on the results of these preliminary experiments, Chelex-100 resin was used for later experiments.

The content of heavy metal-binding organic compounds (HMBOs) in media before or after growth was determined by labeling the medium with ^{63}Ni, passing a 25-ml sample past the resin plug and counting the input and effluent activity. The effluent was sampled at the c. 10 ml volume (mid-sample). Using this procedure, it was possible to chromatograph multiple samples without intermediate washes. When the resin was approaching exhaustion, a band of green color (due to the Ni ion) appeared at the head of the resin plug. The molarity of HMBO in the test solutions was calculated by multiplying the solution Ni molarity by the ratio of effluent to input isotope activities. Only isotope ions bound to organic ligands of effective binding constant comparable to or greater than that

of the iminodiacetate chelating groups of the Chelex-100 resin (pK=18-20) passed through the column. Thus, this technique was selective for organic ligands of the highest avidity.

Trace Metal Analysis

The metal content of solids, such as medium components or bacterial cells, was analyzed by wet ashing (Passman, 1977). Liquid samples were analyzed by two techniques. The first was a liquid-liquid extraction technique using the transition metal-specific chelator extracted into methyl isobutyl ketone (MIBK-APDC) (Conrad, 1974; Parker, 1972; Stolzberg, 1975). Prior to extraction samples were adjusted to pH 6.0 (Conrad, 1974) with 0.2 M pH 6.0 phosphate buffer. Extracts were stored at -96°C if analysis needed to be delayed.

Alternatively, liquid samples were analyzed by the resin extraction technique using Chelex-100 resin (Riley and Taylor, 1968). Resin was prepared by the technique of Davey et al. (1970). The prepared resin was equilibrated to a final pH of 7.9 in QH_2O, and then with 75 % KASW at the same pH. Resin was poured as a 15 X 250 mm bed in an acid-washed chromatographic column. Samples were passed through the column at 22.5 ml/min. The column was eluted with a 50-ml volume of 1 N HNO_3 followed by two washes with 25-ml volumes of QH_2O. The eluate and washes were pooled, neutralized to pH 6.5 with saturated KOH, and rechromatographed onto a 15 X 30 mm short column of Chelex-100 which was reeluted into a 10-ml total volume.

For all samples atomic absorption spectrophotometry was performed on a Varian AA-4 (Varian-Techtron) or IL 351

(Instrument Laboratories) spectrophotometer using the procedures recommended by Varian (Parker, 1972).

Slide Culture

The slide microculture technique of Ensign and Wolfe (1964) was adopted to allow the observation of the growth and morphogenesis of A. marinus on the microscope stage. Several drops of hot agar medium were placed onto warmed sterile microscope slides. The combination of a warmed slide and hot agar allowed the agar to spread as a thin layer. It was vital that the agar layer was as thin as possible, especially if phase contrast microscopy was used, as the quality of the image and its contrast were degraded severely by even a thin layer of clear agar. The agar film was trimmed to a square of c. 15 mm sides with a sterile single-edge razor blade. The block surface was inoculated with 10 µl of a barely turbid cell suspension which was spread using the bent tip of a sterile pasteur pipet. This procedure gave c. 5 - 10 cells per oil immersion objective field. The block surface was covered with a sterile 22 mm coverslip and the edges sealed with sterile melted vaspar. Touching each edge of the coverslip with a briefly flamed bent glass rod gave thorough sealing which was important in prolonged incubations. This procedure left one or more air pockets under the coverslip which were useful for identification of the surface of the agar for focusing. A. marinus was inhibited rapidly due to diminished O_2 tension under the impermeable glass coverslip. The procedure therefore was modified using a method by Noller and Durham (1968)

replacing the glass coverslip with a sterile piece of Yellow Springs Instrument Co. Teflon oxygen probe membrane which was readily permeable to O_2 but impermeable to H_2O.

M9 Nutrient Assay

Glucose was measured by the Worthington Biochemical Glucostat reagent, using the manufacturer's instructions. $PO_4^{=}$ was measured by a molybdate heteropolyblue technique (Boltz and Lueck, 1958). Ammonium was measured by a direct nesslerization technique (Boltz and Lueck, 1958). The same nutrient stocks used to prepare the medium were used to prepare standard solutions.

Taxonomy

With few exceptions the techniques used were those of Colwell and Wiebe (1970). The following media were prepared according to manufacturer's instructions, or by the procedures of the Manual of Microbiological Methods (1975): DNAse agar (BBL), thioglycolate broth (BBL), Trypticase-Soy Agar with 5 % sheep blood, TSI agar slant (BBL), malonate broth (Difco), lysine decarboxylase broth (Difco), MacConkey agar (Difco), EMB agar (Difco), urease agar (Difco), and litmus milk (Difco).

In addition to the MOF medium of Colwell and Wiebe (1970), the original medium of Hugh and Liefson (1953) was prepared.

The arginine dihydrolase test medium of Thornley (1960) was prepared.

The ability of the study organisms to grow anaerobically was assayed in several ways. Cultures in 2216E MOD agar and broth were cultured in a BBL gas pack jar under anaerobic conditions. Cultures were grown in thioglycolate broth, as stab cultures in deep tubes of 2216E MOD agar, and shake tube cultures in the same medium. The growth of the organisms below the surface of the agar was indicative of anaerobic growth. The fermentation medium F-1 of Baumann, Baumann, and Mandel (1971) determined the ability of the organism to grow and produce acid and gas from glucose anaerobically.

The accumulation of the intracellular reserve product poly B-hydroxybutyrate (PHB) was assayed by the technique of Stanier, Palleroni, and Doudoroff (1966).

The induction of cortical hypertrophies in plant tissues was studied by the carrot root disk assay of Lippincott and Lippincott (1969).

An antibiotic specific for members of the Aeromonas-Vibrio group of organisms, O/129 (2,4 diamino, diisopropryl-pteridine dihydrochloride, Calbiochem), was used to differentiate them by the procedures of Colwell and Wiebe (1970). In order to quantitate the level of resistance, the O/129 was made up as an 0.905 µg/ul stock solution in QH_2O, sterilized by pressure filtration through a 0.22-um Millipore filter. Absorbent paper disks were cut from Millipore filter support disks with a 8-mm diameter cork borer and sterilized at 121°C for 15 min. Plates of TSA were spread with the test organisms and sterile disks placed on the plates. Onto the disks was pipetted 1, 2, 5, 10, 20, or 50 µl of the O/129 stock solution

giving 0.9, 1.9, 4.5, 9, 18, and 45 µg per disk. After growth appeared on the plates (24-48 h), the zone of inhibition diameter was read in mm.

The data generated by the Colwell and Wiebe (1970) "Core Characteristics" were key-punched onto computer cards according to their coding scheme. The data was submitted to Brian Austin for analysis using the taxonomic data base of R. R. Colwell at the University of Maryland.

Statistical Analysis

Data was analyzed using the Statpack program set of the UNH DEC-10 computer, manufacturer and experimenter supplied programs for the Monroe Model 324 Programmable Calculator, and the internal programs of a Texas Instruments Model SR-51-II calculator. For statistical analyses, the text of Steel and Torrie (1960) was used as a source for experimental design and tables of statistical values.

CHAPTER IV

RESULTS

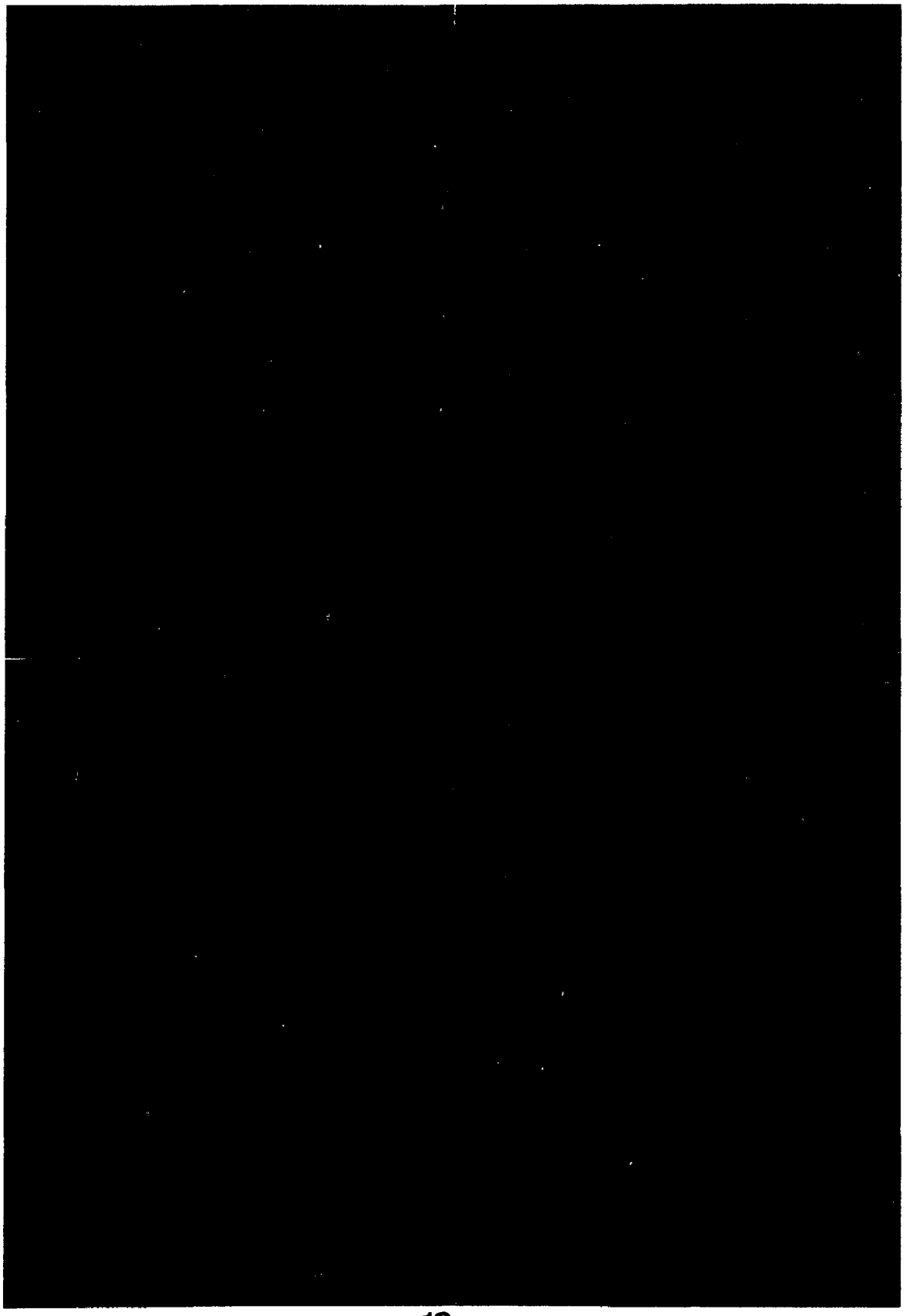
Taxonomy

Gram Stain

The bacterium studied was identified by Cobet, Wirsen, and Jones (1970) as a member of the genus Arthrobacter in large part on the basis of the detection of a cycle of cellular morphology and gram stain reaction. Arthrobacter cells are coccoid and gram-positive in older cultures and develop into irregular pleomorphic gram-negative rods when inoculated into fresh medium. By contrast, Baumann et al. (1972) identified the same bacterium as a member of the genus Pseudomonas. No stage of growth showed gram-positive staining of the cell or granules.

To clarify these conflicting results, the morphology and gram stain reaction of Arthrobacter marinus LAB, P. marina (the independently isolated strain identified by Baumann et al., 1972, as conspecific with Arthrobacter marinus), Arthrobacter crystallopoietes and Arthrobacter globiformis were grown in three media: Trypticase-Soy Broth, 2216E MOD broth, and TRIS-M9 broth. Fig. 2a shows Arthrobacter crystallopoietes grown in 2216E MOD broth. Note the pleomorphy, pseudo-branching, and gram-variability, all characteristic of Arthrobacter. These observations were confirmed in the other two media.

Fig. 2. Arthrobacter crystallopoietes (a), Arthrobacter marinus (b, c) and P. marina (d) grown in 2216E MOD medium at 25°C for 24 h (b), 72 h (d), 360 h (a, c). Gram stain preparations, 1000 X magnification. Camera color balance malfunction caused excessive blue color in b.


10 µm

These characteristics were contrasted with the cell morphology and gram staining shown by Arthrobacter marinus (Fig. 2b and c) and P. marina (Fig. 2d) which failed to show gram-positive cells or granules at any stage of growth. There was definite bipolarity in the uptake of the saffranin counter stain, particularly in younger cultures. These polar-staining bodies may have been what Cobet, Wirsen, and Jones (1970) identified as gram-positive granules. My examination concluded that they were not gram-positive granules or cells. Further, the prominence of such granules in young cells was opposite to the pattern of gram-stain variation in Arthrobacter where older cells are gram-positive.

There was a distinct change in cell shape during growth of Arthrobacter marinus, young cells being rods of 1 X 2 μm and older cells becoming smaller and somewhat coccobacillary, 0.75 X 1.25 μm (Fig. 2b and c). This pattern was unlike that of other Arthrobacter and was characteristic of many gram-negative bacteria. It was concluded that Arthrobacter marinus demonstrated neither the gram stain nor the morphological characteristics of that genus and should be placed in another gram-negative genus.

Flagellation

Cobet (1968) had indicated that Arthrobacter marinus had the degenerate peritrichous flagellation of Conn, Wolfe, and Ford (1940), on the basis of electronmicrographs showing a single, polar flagellum. Baumann et al. (1972) found Arthrobacter marinus was non-motile but its conspecific strains

had 2 to 5 flagella in a broadly dispersed polar tuft. From their discussion, it was unclear whether Arthrobacter marinus was examined for flagella or was screened out on the basis of the absence of wet mount motility.

Motile strains of the P. marina cluster showed pseudoperitrichous insertion of flagella in Leifson-stained preparations due to looping of the flagella back along the cell soma (Baumann et al., 1972). The flagella were polar when observed by electron microscopy.

In the present study, both the ATCC and LAB strains of Arthrobacter marinus were distinctly motile, though often as few as 10 % of the cells of a wet mount were actively motile. Curiously, P. marina failed to show wet mount motility, but both cultures were motile in 0.5 % agar. Flagellation of these cultures using Leifson stain is shown in Fig. 3 and 4. Fig. 3a and b showed apparently polar flagellation of Arthrobacter marinus, while Fig. 3c and d showed cells with apparently peritrichous insertions. Fig. 4 (b, c, d) showed a similar range of insertion patterns for P. marina. Indeed, cells of both strains were found which lacked flagella, or were flagellated polarly, laterally, and peritrichously. Flagella frequently showed formation of loops and coiled forms (Fig. 3a and b, 4b and c). As Leifson (1960) emphasized, such flagella frequently are ineffective for propulsion. This may explain the variation of the wet mount motility findings between the present study and those of Baumann et al. (1972).

In order to determine whether the flagellar insertion pattern described by Baumann et al. (1972) was characteristic

Fig. 3. Leifson stains of Arthrobacter marinus grown in 2216E MOD broth, 25°C, 24 to 72 h.

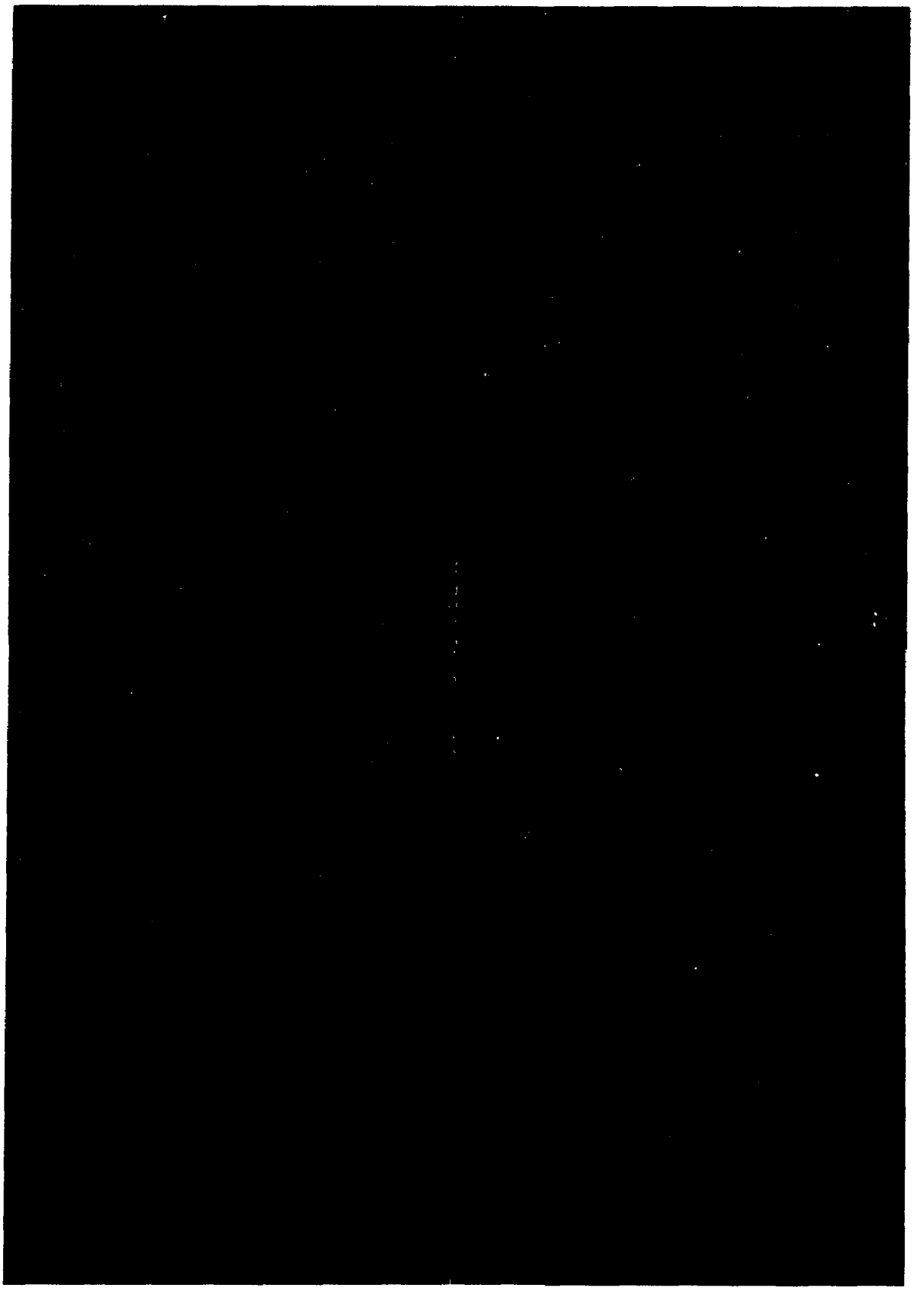
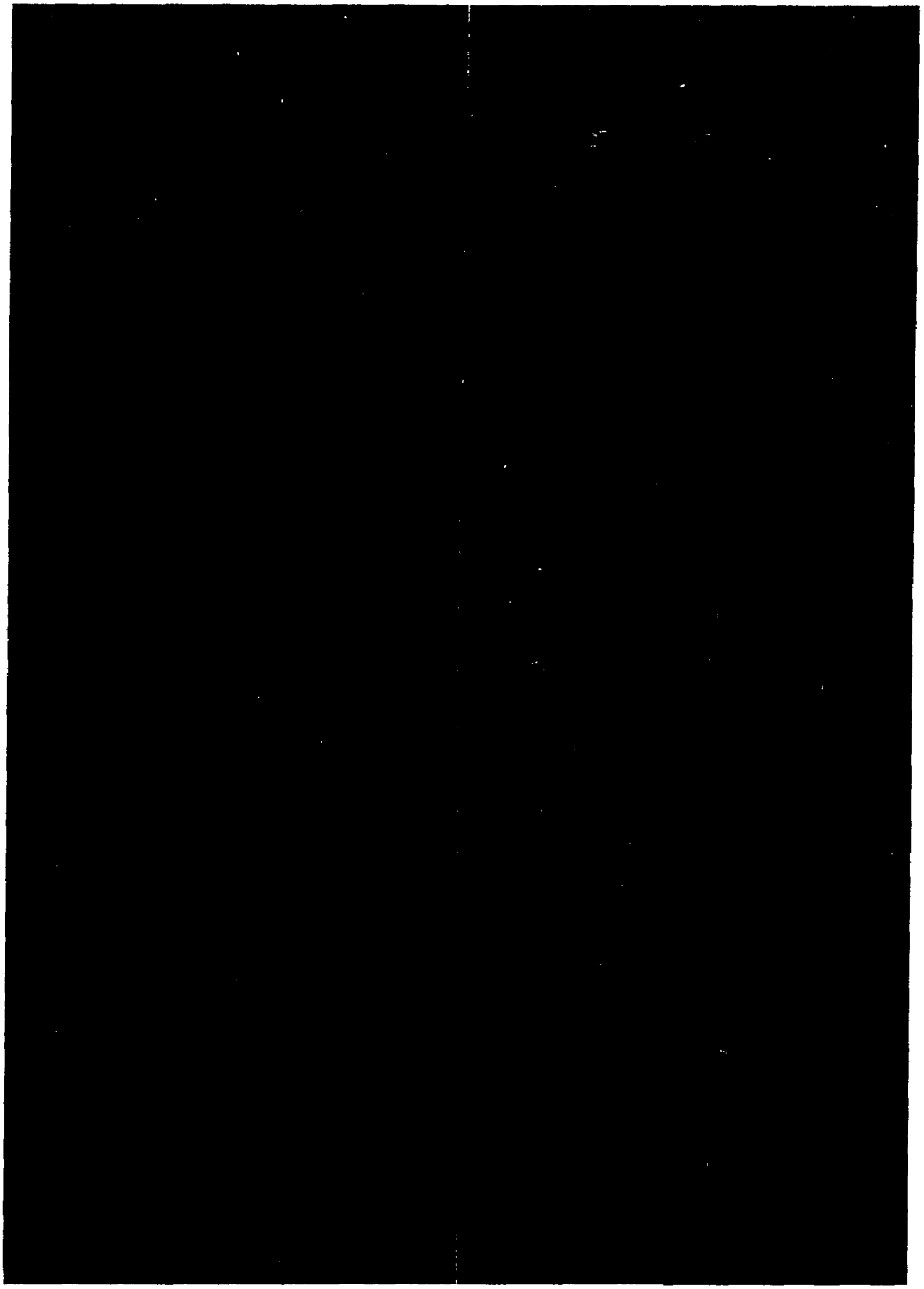

10 µm

Fig. 4. Arthrobacter marinus grown in 2216E LN with 2 X 10^{-4}M Ni, 25°C, 24 h, (a) and P. marina grown in 2216E MOD 25°C, 24 to 72 h (b, c, d).


10 µm

of Arthrobacter marinus, electron microscopy of negatively stained whole cell mounts was used. In a random field a variety of flagellar insertions (Fig. 5a) from distinctly polar to apparently peritrichous, were seen, as were polar flagella looping back along the cell soma (Fig. 5b and c) as described by Baumann et al. (1972). A variety of patterns, from lateral tufts (Fig. 5d and 6a), to broadly dispersed polar tufts (Fig. 6b), and distinctly peritrichous insertions (Fig. 6c and d), were observed in Arthrobacter marinus cultures. The formation of the looped forms on Leifson stains were not artifacts but were observed in electron micrographs (Fig. 5c) as well. Note that the cell in Fig. 6a would appear to have a single lateral flagellum by Leifson stain. The same pattern of flagellation was seen with P. marina. Both P. marina and Arthrobacter marinus were thus flagellated peritrichously.

Gonye (1972) observed multiple, peritrichous flagellation of Arthrobacter marinus under Ni stress and proposed a polar monotrichous to multiple peritrichous shift as a result of Ni toxicity. Preparation of the enlarged and osmotically fragile cells formed by Arthrobacter marinus (termed megalomorphs) under Ni stress for either Leifson staining or negative stained electron microscopy was difficult and rarely successful. However, some micrographs showing small megalomorphic cells with flagella were obtained (Fig. 4a). Such cells were peritrichous but no Ni stress was necessary to explain the observation.

Culture Tests

The cultural tests of the four strains of Arthrobacter marinus, P. marina, P. putida, Alteromonas haloplanktis, P. 130,

Fig. 5. Arthrobacter marinus grown in 2216E MOD 24 to 72 h at 25°C, on formvar substrates, negatively stained with potassium phosphotungstate (b, c) or uranyl acetate (a, d). Magnifications: a (2,720X), b (29,130X), c (11,770X), d (9,280X).

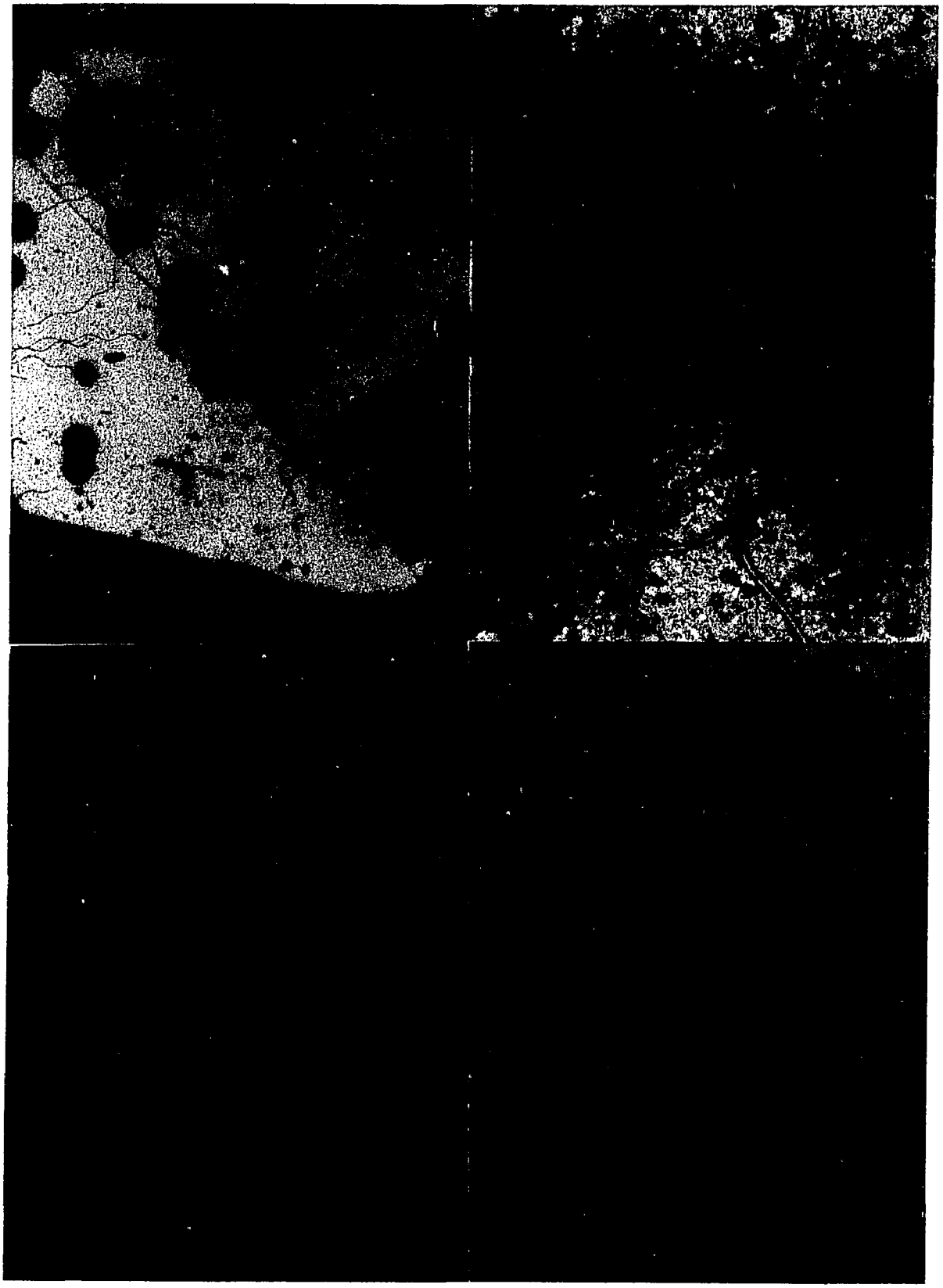
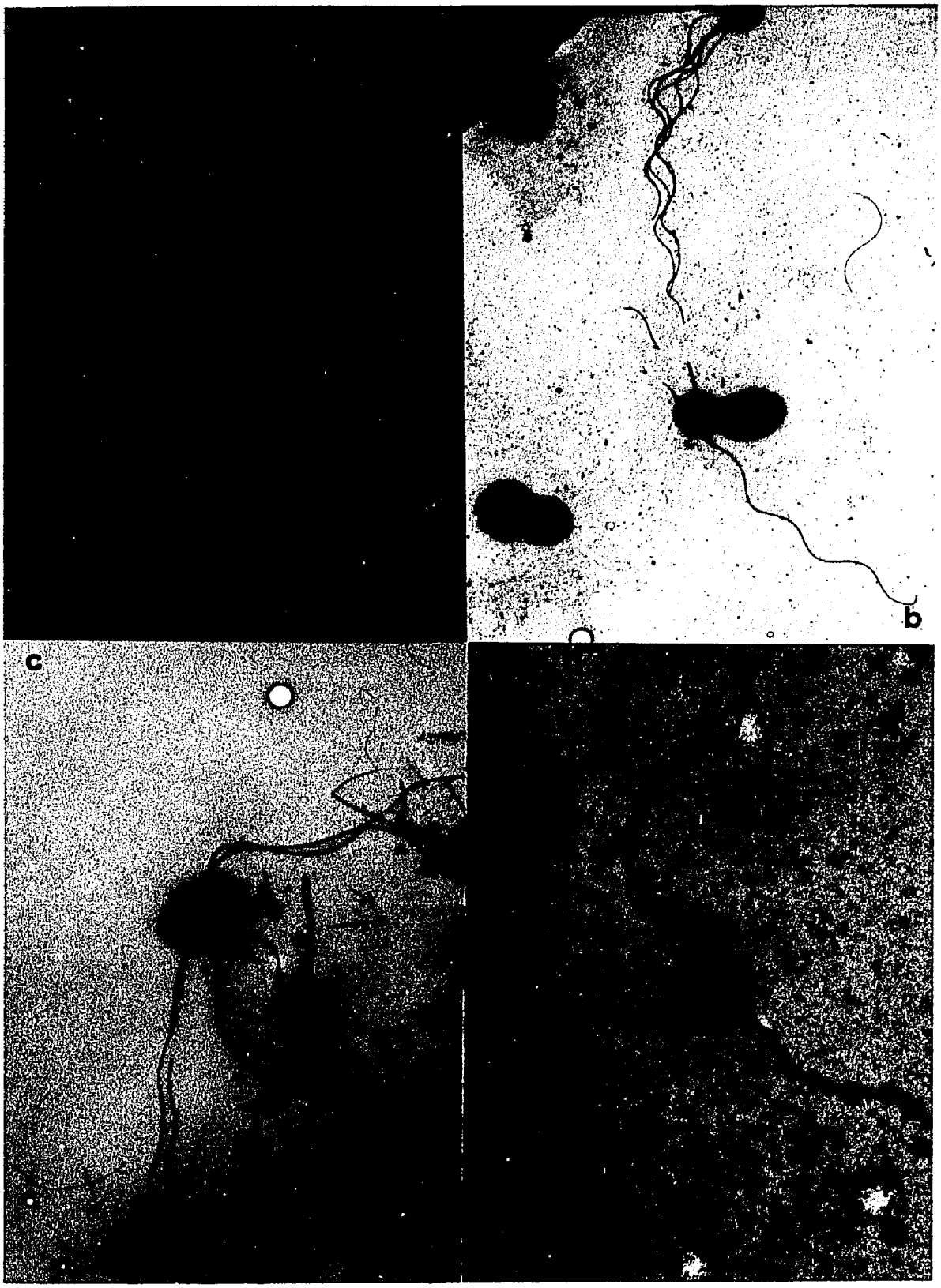


Fig. 6. Arthrobacter marinus grown in 2216E MOD, 24 to 72 h, at 25°C, on formvar substrates, negatively stained with uranyl acetate. Magnifications: a (6,990X), b (5,050X), c (12,410X), D (7,100 X).


b
c

Arthrobacter globiformis, and Arthrobacter crystallopoietes are presented in Table 6. Arthrobacter marinus had broad ranges of temperature and pH tolerance; generally lacked diffusible hydrolytic enzymes; was negative for oxidase, arginine dihydrolase, and amino acid decarboxylases; and was versatile with respect to its nutrition. The metabolism was aerobic and oxidative.

Comparison of Arthrobacter marinus with the two established Arthrobacter cultures showed differences for a number of traits. The lipopolysaccharide test (Limulus amebocyte lysate coagulation) which detects the lipopolysaccharide characteristic of the outer gram-negative cell wall layer was positive for Arthrobacter marinus but negative for the two established Arthrobacters indicating distinct differences in cell wall architecture. Also, note the difference in sensitivity to the antibiotic O/129. Further study showed a variety of Arthrobacter species were sensitive to this antibiotic while other bacteria including two coryneform species presumably related to Arthrobacter (Keddie, 1974) were of lower or no sensitivity (Table 7). Thus, the O/129 sensitivity test may be of value in the differentiation of this genus.

Adansonian analysis of 130 characters of the 10 organisms (Table 8) showed the four strains of Arthrobacter marinus and P. marina to form a tight cluster, linking at 95 % similarity or better. Clearly, Arthrobacter marinus and P. marina form a single species. By contrast, Arthrobacter marinus shows similarity to the tested Arthrobacter species of 56 to 62 %. "Arthrobacter" marinus is not an Arthrobacter.

Table 6. Cultural and biochemical tests of 1) P. putida ATCC 29735, 2) Alteromonas haloplanktis, 3) Arthrobacter marinus LAB, 4) Arthrobacter marinus ATCC, 5) Arthrobacter marinus R-1, 6) Arthrobacter marinus R-2, 7) P. marina, 8) Arthrobacter crystallopoietes, 9) Arthrobacter globiformis, 10) P. 130.

Test	1	2	3	4	5	6	7	8	9	10
Size (μm)	1X2-5	1.25X3	1X1.5	1X1.5	1X1.5	1X1.5	1X1.5	1 X 3	0.75X2.5	1 X 2
Shape	rods	rods	rods	rods	rods	rods	rods	sl rods[1]	pl rods[2]	rods
Arrangement	single	short chains	single	single	single	single	single	single	variable chains	single
Flagellation	1-3 polar	1 polar	2-7 perit[3]	1-5 perit	1-5 perit	1-5 perit	1-5 perit	-	-	1-2 polar
Gram Stain	-	-	-	-	-	-	-	+	+	-
Spores	-	-	-	-	-	-	-	-	-	-
Fat droplets	-	-	+	+	+	+	+	-	-	-
Metachromatic staining	-	-	-	-	-	-	-	-	-	-
Wet mount motility	+	+	+/-[4]	+/-	+/-	+/-	-	-	-	-
Colony shape	convex	convex	raised	raised	raised	raised	convex	convex	convex	raised
Colony size (mm, diameter)	1	2	2 - 5	1	3	2	2	1.5	1	2 - 5
Colony edge	entire	entire	entire	entire	entire	entire	entire	entire	entire	entire

[1] slender rods [2] pleomorphic rods [3] peritrichous [4] weak motility or low fraction motile

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
Colony density	TL[5]	TS[6]	TL	TL	TL	TL	TL	TL	TL	TL
Pigment 2216 E MOD	OW[7]	OW	OW	OW	OW	OW	OW	OW	OW	OW
Pigment P. F agar	YG-Dif[8]	-	-	-	-	-	-	-	-	-
Pigment P. P agar	-	Br-Dif[9]	-	-	-	-	-	-	-	-
Luminescence	-	-	-	-	-	-	-	-	-	-
Broth turbidity	Mod[10]	Mod	Mod	Mod	Mod	Mod	Mod	Mod	Mod	Mod
Broth surface growth	-	-	Pel[11]	Pel	Pel	Pel	Pel	-	-	-
Temperature range, °C	7-30	1-30	1-40	1-40	1-40	1-40	1-40	3-30	10-35	1-35
pH range	4.5-9	4.5-9	4.0-9	4.0-9	4.0-9	4.0-9	4.0-9	5.0-9	5.0-9	5.0-9
Salt requirement	-	Na	-	-	-	-	-	-	-	Na
Nitrate reduction	-	-	-	-	-	-	-	to NO_2	-	-
Relationship to air	Aer[12]	Aer	Aer	Aer	Aer	Aer	Aer	Aer	Aer	Aer
Hugh & Leifson dextrose	Ox[13]	Ox	Ox	Ox	Ox	Ox	Ox	Ox	Ox	Ox

[5] translucent [6] transparent [7] off white [8] yellow-green, diffusible

[9] brown diffusible [10] moderate [11] pellicle [12] aerobic

[13] oxidative metabolism

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
Litmus milk	A[14]	R[15]	R	R	R	R	R	R-Pep[16]	NR[17]	NR
PHB accumulation	-	-	+	+	+	+	+	-	-	+
Lipopolysaccharide test	+	+	+	+	+	+	+	-	-	-
Soft agar motility	+	+	+	+	+	+	+	-	-	+
Growth in NO_2 glucose	+	-	+	+	+	+	+	+	+	-
Growth in NH_4 glucose	+	+	+	+	+	+	+	+	+	+
Urea hydrolysis	-	+	-	-	-	-	-	+ slow	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	-	-	-	-	-	-	-
Diffusible enzymes:										
starch hydrolysis	-	-	-	-	-	-	-	-	-	-
agar hydrolysis	-	-	-	-	-	-	-	-	-	-
gelatin hydrolysis	-	+	-	-	-	-	-	-	-	+
DNAse	-	-	-	-	-	-	-	-	-	-
casein hydrolysis	-	-	-	-	-	-	-	-	-	-

[14]alkaline [15]reduced [16]peptonized [17]no reaction

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
Penicillinase	-	-	+	+	+	+	+	-	-	-
Lecithinase	-	-	-	-	-	-	-	-	-	+
Lipase:Tween 20	-	-	-	-	-	-	-	+	DNG[18]	-
Tween 40	-	+	-	-	-	-	-	+	DNG	-
Tween 60	-	+	-	-	-	-	-	+	DNG	-
Tween 80	-	-	-	-	-	-	-	+	DNG	-
Phosphatase	-	+	+	+	+	+	+	-	-	+
Antibiotic sensitivities:										
penicillin	NS[19]	NS	S[20]	S	S	S	S	S	S	NS
chloromycetin	NS	S	S	S	S	S	S	S	S	S
tetracycline	NS	NS	NS	NS	NS	NS	NS	NS	S	NS
dihydrostreptomycin	NS	S	S	S	S	S	S	NS	NS	NS
colimycin	S	S	NS	NS	NS	NS	NS	NS	NS	S
0/129	NS	NS	NS	NS	NS	NS	NS	S	S	NS

[18] did not grow

[19] not sensitive

[20] sensitive

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
Acid from glucose	+	-	+	+	+	+	+	-	-	-
galactose	+	+/-	+	+	+	+	+	-	-	+
mannitol	-	-	+	+	+	+	+	-	-	+
ribose	-	-	+	+	+	+	+	-	-	-
sucrose	-	-	+	+	+	+	+	-	-	-
lactose	-	-	+	+	+	+	+	-	-	-
NH_3 from peptone	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	+	+	-	+/-	-	-	-	-	-	-
Amino acid decarboxylases:										
lysine	-	-	-	-	-	-	-	-	-	-
ornithine	-	-	-	-	-	-	-	-	-	-
arginine	+	-	-	-	-	-	-	-	-	-
Sole carbon sources:										
carbohydrates -										
glucose	+/-	+/-	+	+	+	+	+	+	+	+
ribose	-	-	+	+	+	+	+	+	-	+
acetate	+	+/-	+	+	+	+	+	+	-	+

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
citrate	+	+/-	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	-	+	+
malonate	+	-	-	-	-	-	-	-	-	-
amino acids -										
alanine	+/-	+	+	+	+	+	+	+	-	+
proline	+	+/-	+	+	+	+	+	+	-	+
glutamate	+	+/-	+	+	+	+	+	+	+	+/-
methionine	-	-	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-
glycine	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
phenyalanine	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-
Carrot root disk assay	-	-	-	-	-	-	-	-	-	-
EMB agar	pink colony	DNG	DNG	DNG	DNG	DNG	DNG	DNG	DNG	DNG
TSI agar slant	A[14]	A	A	A	A	A	A	NR	NR	A
H_2S from peptone	-	-	-	-	-	-	-	-	-	-
H_2S from cysteine	-	+	-	-	-	-	-	-	-	+
H_2S from thiosulfate	-	-	-	-	-	-	-	-	DNG	-

[14] alkaline

Table 6 Continued.

Test	1	2	3	4	5	6	7	8	9	10
Indole	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-
Vogues Proskauer	-	-	-	-	-	-	-	-	-	-
Citrate Simmon's	+	-	+	+	+	+	+	-	-	+
Citrate Koser's	+	-	+	+	+	+	+	+	+	+
Blood agar hemolysis	-	-	-	-	-	-	-	-	-	-
Autotrophic growth on H_2	-	-	-	-	-	-	-	-	-	-

Table 7. Inhibition of four Arthrobacter species, two coryneforms, B. subtilis, A. faecalis, and Arthrobacter marinus, on Trypticase Soy Agar, as diameter (mm) of inhibition zone due to various concentrations of 0/129.

Test Bacteria	0/129 µg per disk					
	0.9	1.8	4.5	9.0	18	45
Arthrobacter marinus	-[b]	-	-	-	5	7
Arthrobacter terregens	ND[c]	15	22	25	30	34
Arthrobacter globiformis	12	17	22	19	22	26
Arthrobacter simplex	-	10	12	13	16	24
Arthrobacter crystallopoietes	ND	20	23	22	26	26
Bacillus subtilis	-	7[a]	10	10	13	17
Brevibacter linens	-	-	-	-	5[a]	7[a]
Kurthia zopfii	-	-	5	5	8	15
Alcaligenes faecalis	-	-	-	-	5[a]	7[a]

[a]Zone corresponds to zone of antibiotic precipitated in the agar

[b]No inhibition, growth to edge of disk

[c]Not determined

Table 8. Percent similarity matrix for ten test bacteria calculated from 130 characteristics.

		1	2	3	4	5	6	7	8	9	10
1	*Arthrobacter crystallopoietes*	100									
2	*Arthrobacter globiformis*	81.5	100								
3	*Arthrobacter marinus* LAB	62.3	56.1	100							
4	*Arthrobacter marinus* ATCC	70.0	63.1	98.4	100						
5	*Arthrobacter marinus* R1	71.5	60.8	100	98.4	100					
6	*Arthrobacter marinus* R2	71.5	60.8	100	99.2	100	100				
7	*Pseudomonas marina*	70.8	64.6	95.4	96.1	95.4	95.4	100			
8	*Pseudomonas cuprodurans*	65.4	63.1	78.5	77.7	73.1	74.6	74.6	100		
9	*Alteromonas haloplanktis*	63.8	63.8	62.3	68.5	67.7	67.7	67.7	76.9	100	
10	*Pseudomonas* sp. 130	68.5	69.2	70.0	68.5	69.2	69.2	70.0	68.2	83.1	100

Arthrobacter marinus linked with the Pseudomonas and Alteromonas species at 78 % or lower.

Table 9 compares the results of the current investigation with the results of the three previous workers on this bacterium. Wirsen (1966) reported a different pH range. Cobet (1968) demonstrated H_2S production. Baumann et al. (1972) found different results on N-free medium (-), sucrose (-), malonate (-), and phenylalanine (-) as sole sources of carbon, and arginine dihydrolase (+). The major disagreement was the motility and flagellation already discussed. Comparison of the data generated by the Colwell and Wiebe (1970) "core characteristics" tests with 230 strains of metal-resistant bacteria from Chesapeake Bay (Austin et al., 1977) found that Arthrobacter marinus failed to cluster with any of those strains (Austin, personal communication).

On the basis of the tests performed Arthrobacter marinus and P. marina comprised two strains of a single species. They were assigned to the genus Alcaligenes as A. marinus. The organism matched the current description of the genus (Hendrie, Holding, and Shewan, 1974) with the exception of the oxidase reaction. The combination of characters in Table 10 failed to match the description of any of the named Alcaligenes strains, and is thus proposed as a separate species, A. marinus.

Ni Resistance and Megalomorphy

The concentration of Ni inhibitory to A. marinus was compared to that for a number of other bacteria in order to determine if the differential Ni resistance of A. marinus was

Table 9. Descriptions of Arthrobacter marinus and Pseudomonas marina compared with current study results.

Test or Observation	Arthrobacter marinus			Pseudomonas marina	
	Wirsen (1966)	Cobet (1968)	Rake (1978)	Baumann, et al., (1972)	Rake (1978)
Size	1 X 1.5 µm	1.2-1.5X2.0-4.0µm	1X1.5µm	ND[1]	1X1.5µm
Shape	ovoid rods	ovoid rods	ovoid rods	straight rods	ovoid rods
Arrangement	single or pairs	single or pairs	single	single or pairs	single
Motility	- wet mount - 0.5% agar	+ wet mount	+ 0.5% agar + wet mount	+ (5/7)	+ wet mount + 0.5% agar
Flagellation	-	1 sub polar	1-5 mixed	2-5 polar	1-5 mixed
Gram stain	-	variable	- bipolar	-	- bipolar
Pigment	"non pigmented"	"cream"	off-white	"non pigmented"	off-white
Fluorescence	ND	ND	-	-	-
Temperature range °C	4*- 37*	6*- 40	1 - 40	4*- 35	1 - 40
pH range	5.5 - 9.5*	ND	4.0 - 9.0	ND	4.0 - 9.0
Salt requirement	sea water	ND	none	sea water (Na^+)	none
PHB accumulation	ND	ND	+	+	+
Litmus milk	ND	reduced	reduced	ND	reduced
H_2S production	ND	+	-	ND	-
Urea hydrolysis	ND	-	-	ND	-
Relationship to air	ND	ND	aerobic	aerobic	aerobic
Hugh and Leifson dextrose	ND	ND	oxidative	oxidative	oxidative

[1]not done

*maximum or minimum of range tested

Table 9 Continued.

Test or Observation	Arthrobacter marinus			Pseudomonas marina	
	Wirsen (1966)	Cobet (1968)	Rake (1978)	Baumann, et al., (1972)	Rake (1978)
N - source: NH_4^+	ND	+	+	ND	+
NO_3^-	ND	+	+	ND	+
N - free medium	ND	-	+ (very weak)	-	+ (very weak)
Catalase	+	+	+	ND	+
Oxidase	ND	ND	-	-	-
Diffusible enzymes:					
amylase	-	-	-	-	-
agarase	-	-	-	-	-
gelatinase	-	-	-	-	-
dnase	ND	ND	-	ND	-
caseinase	ND	ND	-	ND	-
penicillinase	ND	ND	+	ND	+
lipase	ND	ND	-	-	-
phosphatase	ND	ND	+	ND	+
Antibiotic sensitivities:					
penicillin	ND	ND	S[2]	ND	S
chloromycetin	ND	ND	S	ND	S
tetracycline	ND	ND	NS	ND	NS
dihydrostreptomycin	ND	ND	S	ND	S

[2]sensitive

Table 9 Continued.

Test or Observation	Arthrobacter marinus			Pseudomonas marina	
	Wirsen (1966)	Cobet (1968)	Rake (1978)	Baumann et al., (1972)	Rake (1978)
Antibiotic sensitivities:					
colimycin	ND	ND	NS[3]	ND	NS
0/129 vibriostat	ND	ND	NS	ND	NS
Sole carbon sources:					
Carbohydrates:					
glucose	+	ND	+	+	+
ribose	ND	ND	+	+	+
acetate	ND	ND	+	+	+
citrate	ND	ND	+	+	+
sucrose	+	ND	+	-	+
malonate	ND	ND	-	+ (5/7)	-
Amino acids					
alanine	ND	ND	+	+	+
proline	ND	ND	+	+	+
glutamic acid	ND	ND	+	+	+
glycine	ND	ND	-	-	-
phenylalanine	ND	ND	+	+ (1/7)	+
Arginine dihydrolase	ND	ND	-	+ (3/7)	-
Amino acid decarboxylases	ND	ND	-	ND	-

[3] not sensitive

Table 10. Description of Alcaligenes marinus (Arthrobacter marinus Cobet, Wirsen, and Jones, 1970; Pseudomonas marina Baumann, Baumann, Mandel, and Allen, 1972).

Rods 1.0 - 1.25 x 1.5 - 2.0 μm. Motile with one to five peritrichous flagella. Colony off-white.

Aerobic, oxidative. Produces acid but not gas in peptone medium from dextrose, galactose, mannitol, ribose, sucrose, and lactose.

Ammonium and nitrate utilized as sole source of nitrogen, nitrates not reduced.

A variety of carbohydrates and amino acids are utilized as sole sources of carbon. Chemolithotrophic growth on H_2 not demonstrated. PHB accumulated.

Litmus milk rapidly reduced, no pH change.

Temperature range 1° to 40° C, optimum 30° C. pH range 4.0 to 9.0.

Sea water not required for growth.

Starch, agar, gelatin, casein, DNA, lipid, urea not hydrolyzed. Penicillinase, phosphatase present. Lysine decarboxylase, arginine dihydrolase absent. Oxidase negative, catalase positive.

H_2S not produced from peptone, cysteine, or thiosulfate.

G + C content of DNA 63.0 $\pm$ 0.5%.

Habitat: sea water.

Reference strain ATCC 25374.

sufficiently great to allow its use as a selective trait in studies of the genetics of Ni resistance. Agar plates of Trypticase Soy Agar, Marine Agar, and 2216E LN were prepared with various concentrations of Ni and the organisms streaked onto them. After 72 h incubation at 25°C the highest Ni concentration giving visible growth was recorded (Table 11). A. marinus was more resistant to Ni than a number of the other strains. There were minor differences between the three strains of A. marinus used. Terrestrial bacteria tended to give maximum Ni resistance on Trypticase Soy Agar (distilled water based), while marine isolates gave the greatest resistance on Marine Agar. Although 2216E LN has 0.1 X the nutrients of Marine Agar, and thus stoichimetrically 0.1 X the complexing capacity, Ni resistance in the lower nutrient medium was typically 0.4 X that of the Marine Agar level. Interestingly, the most Ni-resistant bacterium studied on any of the media was Arthrobacter crystallopoietes.

A. marinus gave extremely enlarged, vacuolated cells when stressed by nearly bacteriostatic concentrations of Ni in peptone based media (Fig.15). At the same concentration of Ni, P. marina formed megalomorphs indistinguishable from A. marinus.

The distribution of megalomorph-producing bacteria was determined from Great Bay estuarine water plated onto 2216E MOD agar with and without 1 X 10^{-3}M Ni (Table 12). Approximately 1.3 % of the population which developed on that medium without added Ni was found on plates with 1 X 10^{-3}M Ni. From the Ni containing plates 16 isolates were taken for

Table 11. Inhibitory concentration of Ni for 13 bacteria on three agar media.

Organism	Highest molarity of Ni allowing growth		
	Media		
	TSA[3]	MA[4]	LN[5]
A. marinus LAB[1]	1×10^{-3}[6]	3×10^{-3}	1×10^{-3}
A. marinus R2[1]	1×10^{-3}	2×10^{-3}	7×10^{-4}
A. marinus R1[1]	2×10^{-3}	3×10^{-3}	2×10^{-3}
E. coli CSH 26	4×10^{-3}	3×10^{-3}	2×10^{-3}
P. iodinum	4×10^{-3}	4×10^{-3}	1×10^{-3}
P. putida	4×10^{-3}	1×10^{-3}	5×10^{-4}
E. coli 4BFW26	4×10^{-3}	4×10^{-3}	1×10^{-3}
S. gallinarum	2×10^{-3}	2×10^{-3}	5×10^{-4}
E. coli W1A[2]	3×10^{-3}	2×10^{-3}	5×10^{-4}
Arthrobacter crystallopoietes[2]	8×10^{-3}	4×10^{-3}	4×10^{-3}
E. coli HFR-C	6×10^{-3}	4×10^{-3}	2×10^{-3}
P. putida ATCC 29735[1]	2×10^{-3}	3×10^{-3}	1×10^{-3}
P. sp. 130	2×10^{-3}	3×10^{-3}	2×10^{-3}

[1]marine isolate

[2]soil isolate

[3]Trypticase Soy Agar (BBL)

[4]Marine Agar (Difco)

[5]2216E LN agar

Table 12. Average CFU/ml developing from Great Bay estuarine samples incubated for 5 weeks at 4°C and 2 weeks at 20°C on 2216E MOD agar with and without 10^{-3}M $NiCl_2$.

	Physical parameters			CFU per ml					
Sample date	T, °C	pH	Salinity, %	2216E MOD[b]	+Ni	Ni%[a]	2216E MOD[c]	+Ni	Ni%[a]
7-July-76	21.3	7.92	30.8	$3.6X10^4$	$1.7X10^2$	0.47	$2.4X10^3$	23	0.96
12-July-76	21.0	7.96	31.0	$4.3X10^4$	$3.4X10^2$	0.79	$2.7X10^4$	47	0.96
21-July-76	21.7	7.86	31.0	$8.5X10^4$	$2.4X10^2$	2.82	$2.7X10^3$	47	1.73
					Mean =	1.36%		Mean =	1.22%

[a] % of control

[b] at 4°C

[c] at 20°C

morphological study. The source and characteristics of these isolates are given in Table 13. Note that 11 (68.7 %) of the 16 isolates gave some form of morphological aberration when grown in the presence of Ni. While some gave megalomorphs indistinguishable from A. marinus, others gave bizarre forms with pseudomycelial appearances (Fig. 7). The taxonomy of these isolates was not studied further, but the Ni-induced megalomorphic bacteria (69 % of the Ni-resistant isolates in a small sample) may represent a coherent taxonomic grouping.

Growth Studies

Measurement of Growth

The primary focus of this research was the growth response of A. marinus to toxic concentrations of Ni, with particular focus on the early growth phases, lag and the initiation of growth. It was thus necessary that the method chosen for the measurement of growth be both sensitive and precise. An experiment was performed to monitor the growth of A. marinus in 2216E LN medium, with 0 or 4 X 10^{-4}M Ni added. Five different means of growth measurement were used: OD, total cell count by microscope, CFU by spread plating, dry cell mass, and protein. An attempt to quantitate cell DNA content failed. Single 1500 ml volumes of medium with and without Ni were prepared, inoculated with 1.0 ml of an 18 h starter culture, and the five growth parameters monitored at four times (0 Ni: 0, 6, 8, 11 h; 4 X 10^{-4}M Ni: 0, 8, 11, 24 h). At each sampling time three aliquants were drawn from the primary culture flask, and growth measured independently on

Table 13. Microbial isolates from 2216E MOD agar with 1 X 10^{-3}M Ni inoculated with Great Bay estuarine water incubated at 20°C for 2 weeks, and Ni stressed and unstressed cell morphology.

			Morphology of cells grown on:	
Strain #	Date	Colony Description	2216E MOD	2216E MOD + 1X10^{-3}M Ni
41	7-July-76	translucent, colorless	rods 1 X 2-5 μm	3-6 μm megalomorphs
42	7-July-76	translucent, colorless	rods 0.75X1.5 μm	rods 0.5 X 1.5 μm
43	7-July-76	translucent, colorless	rods 0.75X1.5 μm	?[3]
44	7-July-76	translucent, colorless	rods 0.75X2.5 μm	4-8 μm megalomorphs
46	7-July-76	translucent, colorless	curved rods 0.5X1.25 μm	2-8 μm megalomorphs ps[1]
47	7-July-76	translucent, colorless	cocci 0.75 μm	4-15 μm megalomorphs ps p[2]
49	7-July-76	translucent, colorless	cocci 0.5 μm → rods 0.75 X 3 μm, chains	1 X 5 μm ps
50	7-July-76	translucent, colorless	cocci 0.5 μm → rods 0.75 X 2 μm	1 X 5 μm ps
51	7-July-76	translucent, colorless	cocci 0.5 μm	1 X 2.5 μm
62	21-July-76	translucent, colorless		5-10 μm megalomorphs p
63	21-July-76	translucent, colorless		rods 1 X 3 μm
64	21-July-76	translucent, red		5-10 μm ps
65	12-July-76	opaque, pink	yeast cells	
66	12-July-76	translucent, colorless		ps

Table 13 Continued.

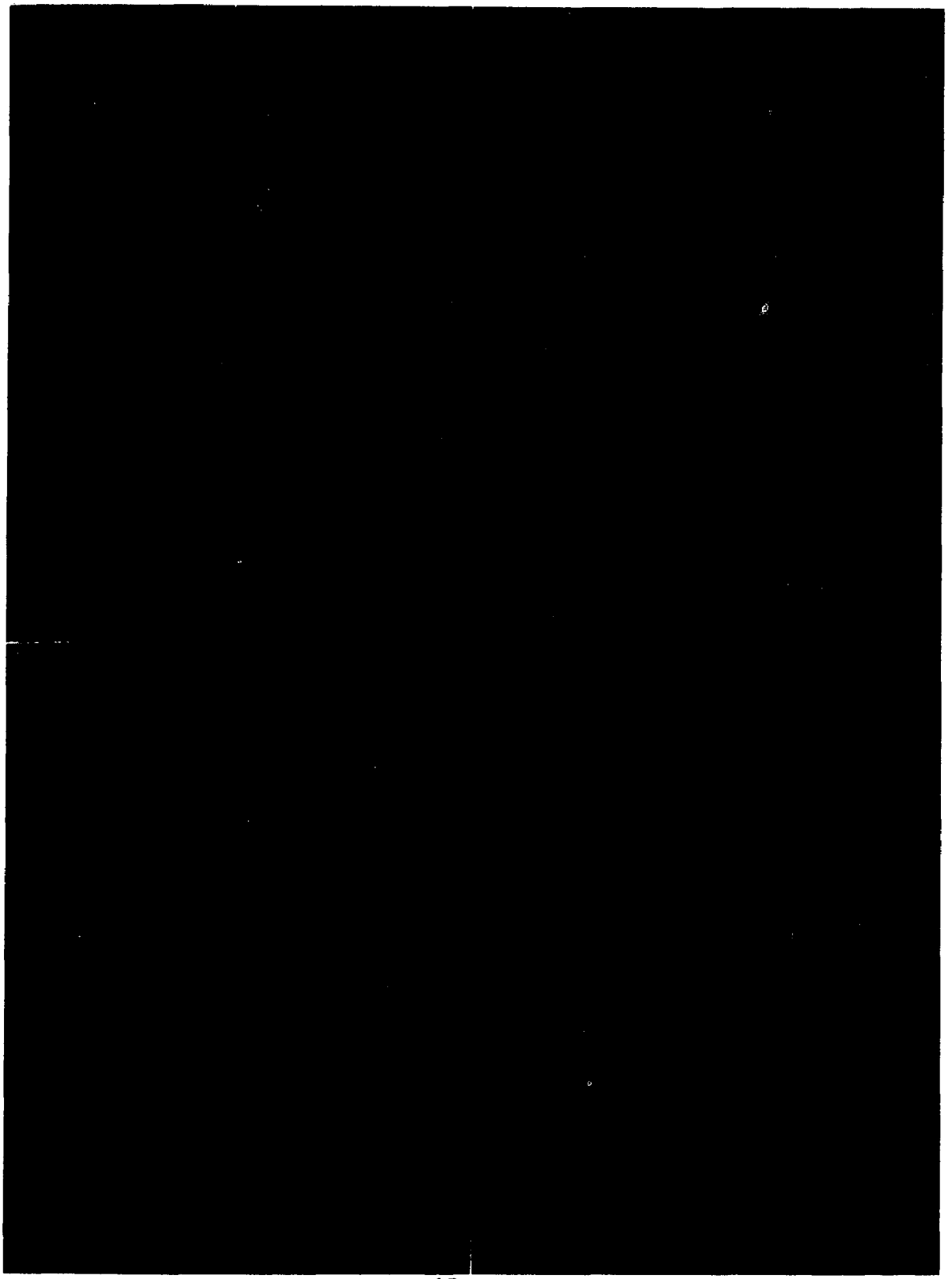
Strain #	Date	Colony Description	Morphology of cells grown on: 2216E MOD	2216E MOD + $1X10^{-3}$M Ni
67	12-July-76	translucent, colorless		5-10 μm megalomorphs
68	12-July-76	translucent, colorless		ps

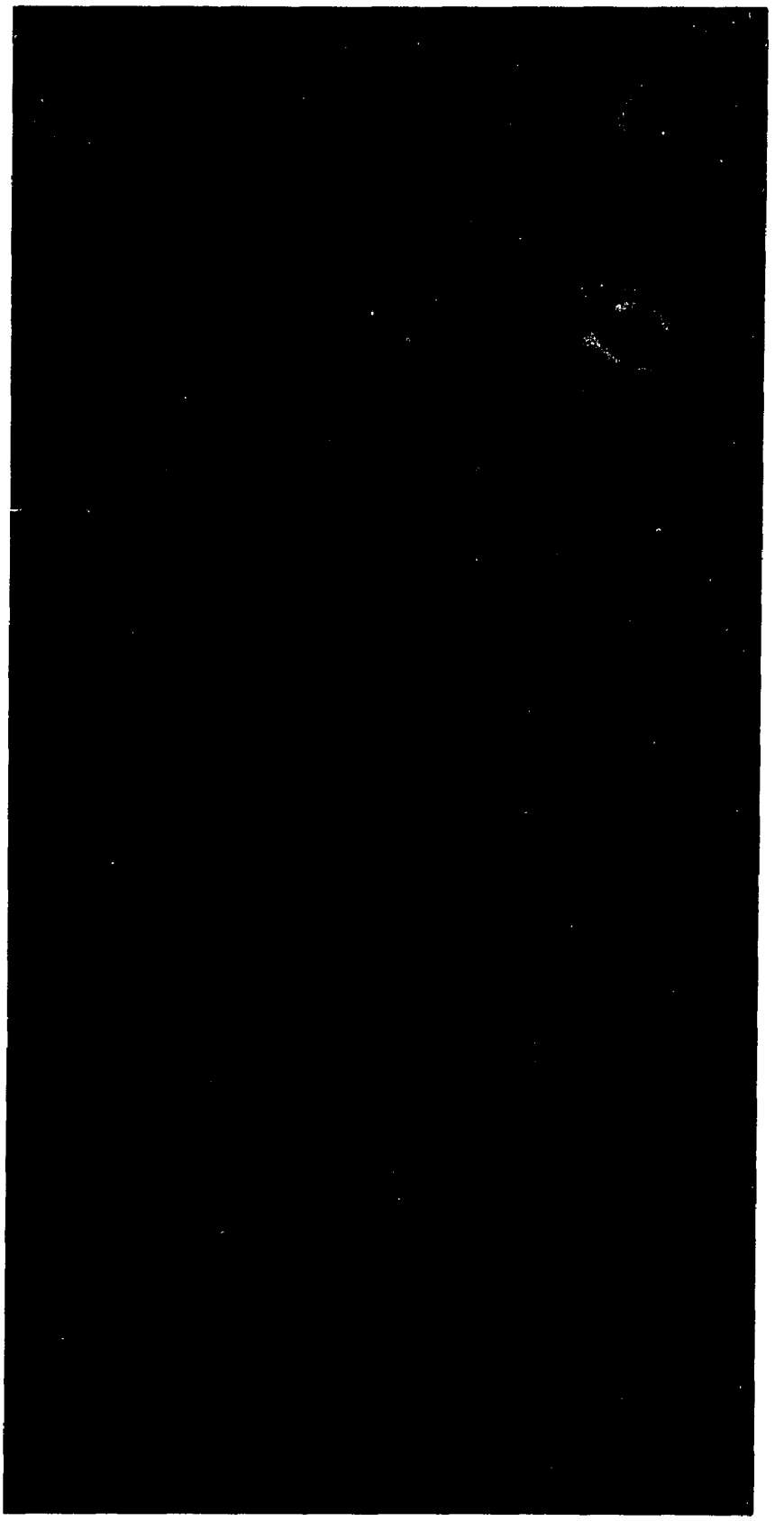
[1]ps = pseudomycelial forms

[2]p = projections

[3]culture lost

Fig. 7. Megalomorphic cells produced by Ni-resistant isolates from estuarine samples (Table 13) when grown on 2216E MOD agar plus 1 X 10^{-3}M Ni: (a) isolate 44, (b) isolate 46, (c) and (d) isolate 47, (e) isolate 49, (f) isolate 41, (g) isolate 51.


10 µm


10 µm

each. The OD of each sub-sample was determined once, the total count twice, the CFU by triplicate plates of two dilutions, and dry weight and protein by 12 filters. Extensive replication was chosen on the basis of a preliminary experiment, using duplicate determinations of each parameter in which random variation made all but the OD values meaningless.

The resulting values, as mean $\pm$ 1 standard deviation, are presented in Table 14. The mean coefficient of variation (standard deviation as percent of the mean) is also given in Table 14 for each parameter. This statistic is a measure of the precision of the determination. The coefficient of variation of the OD was by far the lowest, thus it could be measured with the greatest precision. By contrast, even with the very high degree of replication used the mean coefficient of variation of the other parameters was in the 28 to 50 % range, a very low precision.

In order to determine which parameters best reflected A. marinus growth, the correlation was examined. The correlation coefficient (a statistic descriptive of the linear relatedness of two independent variables) was determined for each of the five parameters plus the log transformations of total count and CFU against each of the other factors. This analysis gave a 7 X 7 matrix of correlation coefficients (i.e. OD versus OD, total count, CFU, cell mass, protein, log total count, and log CFU; total count versus OD, total count, CFU, etc.). For the 0 Ni data all correlations were significant at the 99 or 99.9 % confidence level, indicating that the parameters all increased concurrently. Log transformation of the cell count data did

Table 14. Five growth measurements (mean ± 1 standard deviation) for A. marinus grown in 2216E LN and with 4×10^{-4}M Ni at 4 incubation times.

Ni Concentration, M	Time, h	OD	Total Count, ml	CFU Count, ml	Cell Mass[a] µg/ml	Protein[a] µg/ml
0	0	0.029 ± 0.006	6.5×10^6 $\pm 4.5 \times 10^6$	9.2×10^5 $\pm 5.9 \times 10^5$	62.5 ± 17.7	1.00 ± 0.69
	6	0.153 ± 0.011	3.5×10^7 $\pm 7.2 \times 10^6$	9.8×10^6 $\pm 2.3 \times 10^6$	44.0 ± 10.7	4.70 ± 2.63
	8	0.561 ± 0.013	4.8×10^8 $\pm 2.2 \times 10^8$	7.5×10^7 $\pm 1.5 \times 10^7$	239 ± 13.9	33.0 ± 5.3
	11	1.18 ± 0.011	3.3×10^9 $\pm 1.7 \times 10^9$	2.6×10^8 $\pm 8.6 \times 10^7$	378 ± 199	75.9 ± 22.2
4×10^{-4}	0	0.034 ± 0.004	2.8×10^6 $\pm 1.1 \times 10^6$	2.3×10^6 $\pm 1.8 \times 10^6$	42.4 ± 6.1	1.01 ± 0.53
	8	0.077 ± 0.004	6.5×10^5 $\pm 4.6 \times 10^5$	1.4×10^6 $\pm 6.6 \times 10^5$	71.1 ± 12.5	6.54 ± 3.44
	11	0.155 ± 0.008	1.6×10^6 $\pm 8.9 \times 10^5$	9.9×10^5 $\pm 2.7 \times 10^5$	46.6 ± 30.8	4.80 ± 2.18
	24	0.278 ± 0.005	1.8×10^7 $\pm 1.1 \times 10^7$	1.1×10^6 $\pm 1.2 \times 10^5$	113 ± 13.4	19.1 ± 5.11
Mean Coefficient of Variation ($s/\bar{x} \times 100\%$)		6.62%	51.3%	41.5%	27.5%	43.8%

[a]Corrected for control blanks.

not increase, or decrease the correlation, indicating that the OD, cell mass, and protein were varying directly with cell numbers. By contrast, the 4 X 10^{-4}M Ni data for several parameters had correlations which were below the significance level, particularly the total count, CFU and cell mass parameters, indicating that these parameters were not accurately reflecting changes in the A. marinus biomass.

Table 15 gives the complete linear regression analysis of OD versus the other four parameters for both Ni concentrations. Note the reduced correlation coefficients for the 4 X 10^{-4}M Ni parameters (below significance for CFU). There was good agreement of the regression slopes (m) for OD versus both cell mass and protein at the two Ni concentrations. This indicated that irrespective of the changes in cell size and morphology caused by Ni a unit of turbidity corresponded to a constant amount of cell biomass (OD 1.0 was approximately 280 µg/ml of dry weight or 65 µg/ml of protein). By contrast the ratio of OD to cell number (by either counting means) changed by 40 to 54 fold. The reduced cell count to OD ratio at the higher Ni concentration was due to the increased cell volume of A. marinus cells grown in the presence of Ni. The total count was consistently 7 to 15 times the CFU count. This was due to the propensity of A. marinus to grow in cohesive cell clumps, which were not broken up by vigorous mixing, or spread plating, even when surface active agents were added to the growth medium or diluent solution. When it was necessary to interconvert OD and cell count, mass or protein, the regression

Table 15. Linear regression analysis of growth parameter data.

Regressions	$y = \underline{m}^{d}x$	$\underline{b}^{e}$	$\underline{r}^{f}$
0 Ni			
a. OD vs Total Count/ml	2.87×10^{9}	-4.26×10^{8}	0.928[c]
b. OD vs CFU/ml	2.20×10^{8}	-3.09×10^{7}	0.943[c]
c. OD vs Dry Cell Mass (µg)	291.7	34.62	0.969[c]
d. OD vs Protein (µg)	64.72	-2.89	0.960[c]
4×10^{-4} M Ni			
a. OD vs Total Count/ml	6.43×10^{7}	-3.09×10^{6}	0.703[f]
b. OD vs CFU/ml	4.07×10^{6}	1.99×10^{6}	-0.421[a]
c. OD vs Dry Cell Mass (µg)	273.8	28.57	0.690[b]
d. OD vs Protein (µg)	66.41	-0.954	0.837[c]

[a]Not significant

[b]Significant at the 95 % level

[c]Significant at the 99.9 % level

[d]Regression slope

[e]y-intercept

[f]Correlation coefficient

of slopes for 0 Ni were used. Thus an OD of 1.0 corresponded to 292 µg/ml of dry cells.

Although reservations have been expressed (Chesbro, personal communication) concerning the use of OD as a measurement of culture growth, particularly at low cell densities, it was concluded that of the five growth parameters studied OD was the most useful. The formation of cell clumps made CFU determinations unacceptably imprecise. Thus, an increase of cell mass and numbers in 4 X 10^{-4}M Ni (Table 14) which was detected by OD, cell mass, and protein, was undetectable by CFU. The total count technique, though potentially sensitive to small changes in cell numbers, suffered from poor precision (51 % coefficient of variation) which would obscure small changes in count within the random statistical fluctuation around the mean. The total count technique was also very time consuming, particularly at low cell counts, and was ill-suited to monitoring of large numbers of cultures, or at closely spaced sampling points. Dry cell mass and protein also had poor precision compared to OD, and entailed a time lag between sampling and data acquisition. It was therefore concluded that OD, which was determined rapidly, immediately, and with high precision, was the optimum growth monitoring technique available, and it was used for subsequent growth measurements.

The relationship between OD and dry cell mass was separately determined for A. marinus grown in UB-M9 medium. A 24 h culture of A. marinus in UB-M9 broth (25°C, shaken) was washed once and adjusted to an OD of 15.9. Five aliquants (2 ml) were filtered through tared 47-mm 0.4-µm Nuclepore

filters which were dried at 60°C and reweighed (Mettler H54 balance). Measured net cell mass was 3.315 ± 0.164 mg or 104.2 ± 5.14 µg/OD/ml. The calculated value of 104 µg/OD/ml was one third the value derived above (Table 15; 291.7 µg/OD/ml) for cells grown in 2216E LN medium. Thus an equal mass of UB-M9 grown cells scattered three times as much light as 2216E LN grown cells. The difference was perhaps due to the occurrence in UB-M9 grown cells of brightly refractile (phase contrast microscopy) granules, and the smaller size of UB-M9 grown cells (0.75 X 1.5 versus 1.0 X 2.0 µm).

In the determination of cell uptake of Ni, it was necessary to know the relationship between the dry weight of A. marinus and the cell wet weight. It was impossible to directly measure wet cell weight, as there was no way to remove the excess interstitial water from the cells. It was therefore necessary to determine this relationship by calculation. The shape of the A. marinus cell was approximated as a cylinder capped by hemispheres. Thus a cell 1 X 2 µm (typical 2216E LN grown morphology) would have a cell volume of 0.916 μm^3. Assuming a cell density of 1.0 g/cm^3, and OD 1.0 culture (2.9 X 10^9 cells/ml, 292 µg/ml; Table 15) would have a mass of 2.6 mg/ml. This corresponds to 88 % water in the cells. Thus as an approximation, the wet weight of A. marinus was 8.9 times the dry weight. However, Chesbro (personal communication) indicates 80 % water is a general mean bacterial water content.

Ni Toxicity in Complex Medium

Effect of Inoculum Size

Initial experiments in peptone based media were designed to duplicate and confirm the pattern of response of A. marinus in complex media which had been reported by earlier workers with this organism (Cobet, 1968; Gonye, 1972) and to document growth in complex medium in a format comparable with M9 growth data (below). Preliminary experiments demonstrated a great sensitivity of response to the size of the inoculum. This effect of inoculum size on the pattern of growth response in 2216E LN medium, plus the effect on the pattern of morphological response of the organism, was examined in five experiments set up with added Ni concentrations ranging from 0 to 4 X 10^{-3}M. Inoculum sizes of 0.01, 0.1, 1.0, and 10.0 ml of a washed 2216E LN starter culture were used. A set of flasks was also run at the 0.1 ml EQ inoculum using an unwashed starter culture to test if any carry over from the starter might effect experimental results.

In order to illustrate the differential effect of inoculum size upon the growth response of A. marinus, the data for three of the Ni concentrations at which each of the inocula gave growth are combined in Figs. 8, 9, and 10. At 0 Ni (Fig. 8) there were small effects of inoculum size upon the apparent lag and growth yield of the culture.

At 4 X 10^{-4}M Ni (Fig. 9) the effects were more dramatic. At all inoculum sizes there was a dramatic depression of growth yield, from peak OD values of 1.0 to 1.2 in 0 Ni medium to peak

Fig. 8. Growth response (OD) of A. marinus in 2216E LN broth, shaken, incubated at 25°C, at various inoculum sizes.

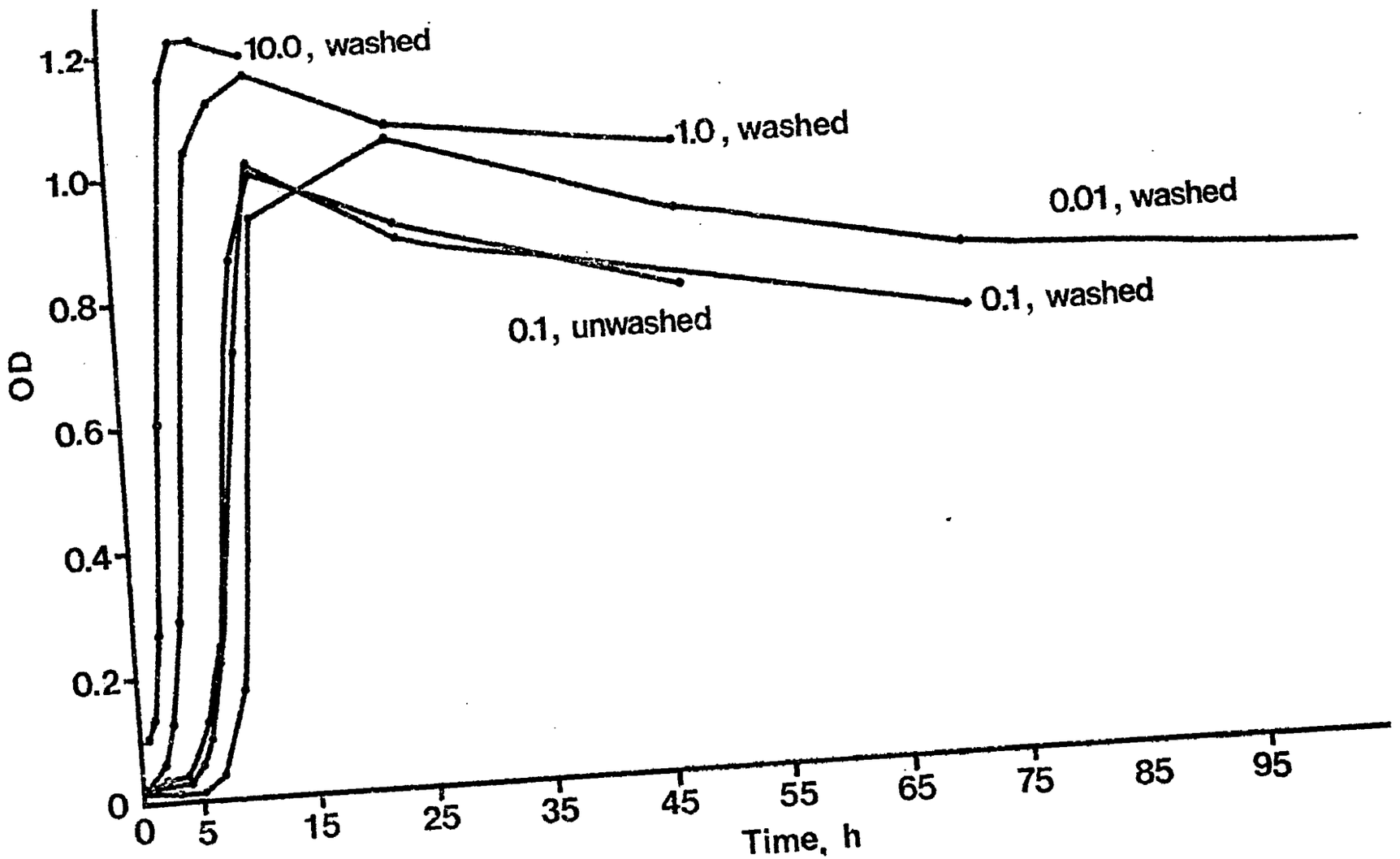

10.0, washed
1.0, washed
0.01, washed
0.1, washed
0.1, unwashed
OD
1.2
1.0
0.8
0.6
0.4
0.2
0
0
5
15
25
35
45
55
65
75
85
95
Time, h

Fig. 9. Growth response (OD) of A. marinus in 2216E LN broth with 4 X 10^{-4}M Ni, shaken, incubated at 25°C, at various inoculum sizes.

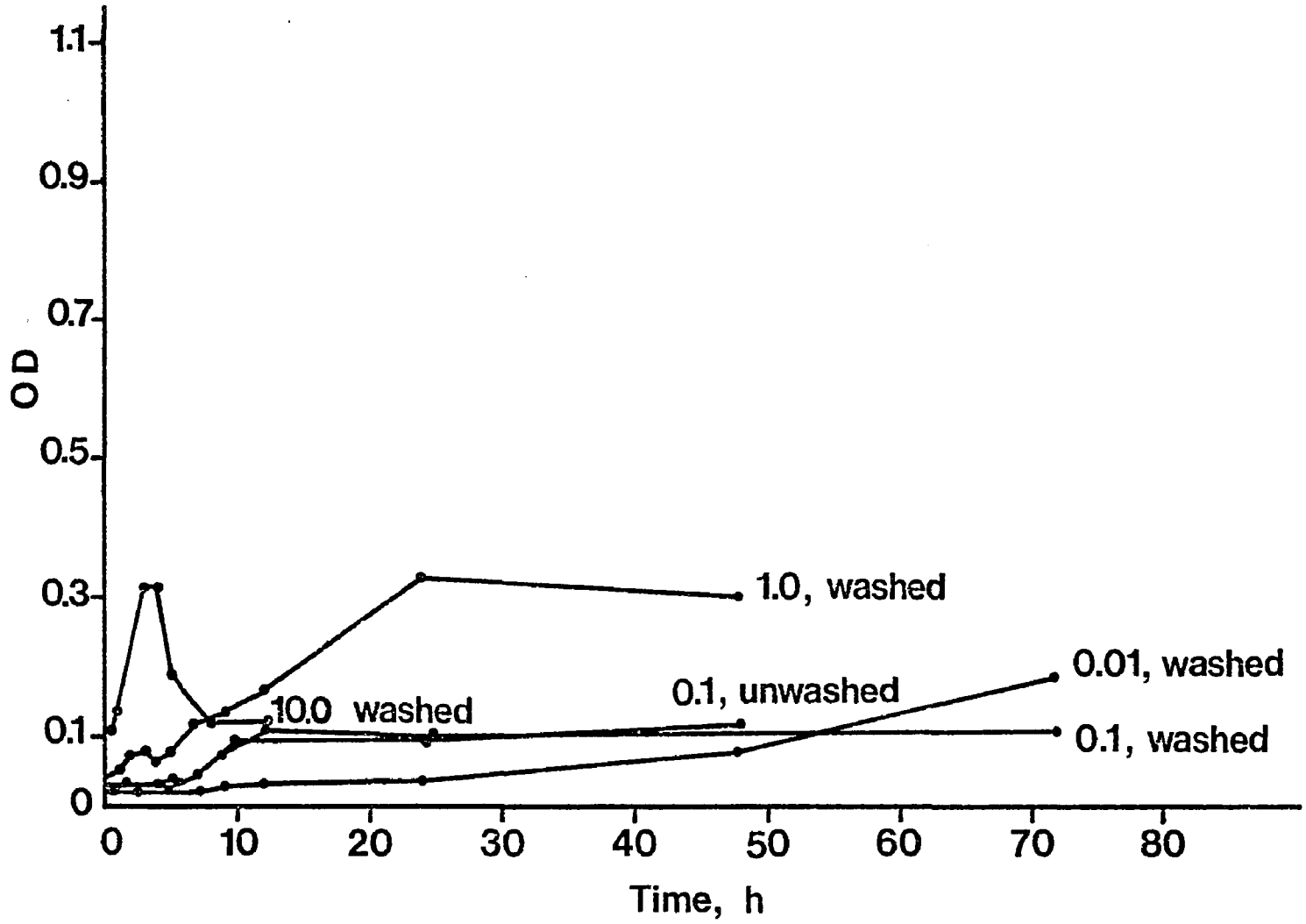
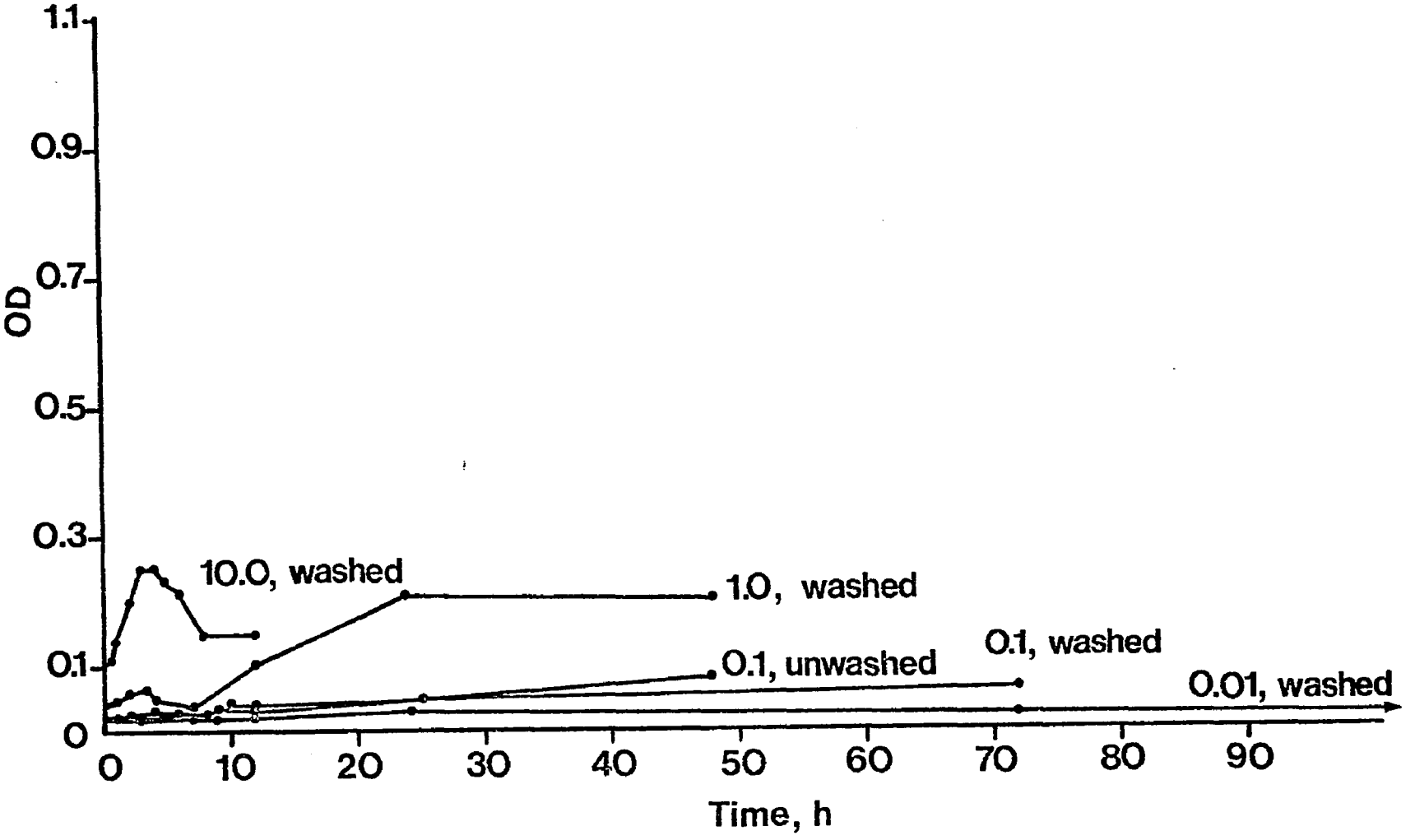

1.1
0.9
0.7
0.5
0.3
0.1
0
O D
0
10
20
30
40
50
60
70
80
Time, h
1.0, washed
0.01, washed
0.1, unwashed
10.0 washed
0.1, washed

Fig. 10. Growth response (OD) of A. marinus in 2216E LN broth with 8 X 10^{-4}M Ni, shaken, incubated at 25°C, at various inoculum sizes.


1.1
0.9
0.7
0.5
0.3
0.1
0
OD
10.0, washed
1.0, washed
0.1, washed
0.1, unwashed
0.01, washed
0
10
20
30
40
50
60
70
80
90
Time, h

OD values of 0.1 to 0.3 in the Ni containing medium. Furthermore, at 0 Ni all inoculum sizes showed essentially the same pattern of growth, but at 4 X 10^{-4}M Ni different inoculum sizes showed distinctly different growth patterns. The 10 ml EQ inoculum showed an immediate induction of rapid growth reaching a peak OD of 0.32 within 3 h. Growth of the 1.0 ml EQ inoculum was much slower, though it appeared to start as soon as the larger inoculum did. It reached approximately the same final OD as the 10 ml EQ inoculum did, but only after 24 h incubation. The 0.1 ml EQ inoculum size (note that here as in 0 Ni the washed and unwashed inocula behaved identically) showed a much briefer period of growth at about the same rate as the 1.0 ml EQ inoculum, which started after a lag of about 5 h (this inoculum size gave a lag of about 4 h in 0 Ni). The growth, when it did occur, shut off abruptly after an OD of only 0.1 was reached. The smallest inoculum (0.01 ml EQ) showed an extended period of what appeared to be slow growth, reaching a maximum OD of nearly 0.2 only after 72 h.

At 8 X 10^{-4}M Ni (Fig. 10) the largest inoculum again showed the early induction of a period of rapid growth, this time peaking at about OD 0.25. Again the 1.0 ml inoculum showed a slower growth, but reached a final OD (0.2) comparable to that in the 10.0 inoculum. At this Ni concentration the flasks receiving the two smallest inocula showed essentially no growth.

Three descriptive growth parameters, doubling time, maximum OD, and lag time, for all Ni concentrations studied at each inoculum size were calculated from the growth data.

The doubling time of all the inoculum sizes (Fig. 11) were nearly identical, at from 1.2 to 1.7 h at 0 Ni. In all cases the doubling time increased rapidly above the point (about 1×10^{-4}M) at which the growth yield, as OD_{MAX}, began to drop (Fig. 12). The ratio of the doubling time at the highest Ni concentration giving any detectable upward OD change, to that at 0 Ni, was typically 40 to 50 fold. The maximum OD remained constant up to a limiting Ni dose (5×10^{-5} to 1×10^{-4}M Ni), which was inoculum independent. This point was considered the minimum inhibitory dose. The OD_{MAX} dropped roughly inversely to the rise in doubling time. By contrast, the lag time, the time between inoculation and the initiation of OD increase, was roughly constant for each inoculum size (Fig. 13), over the range of Ni concentrations. Evidently in 2216E LN medium the lag time was dependent only on the inoculum size. No lag times were given for the highest Ni concentrations of the 0.1 washed and 0.01 ml inoculum sizes, for though there was a slight increase in the OD during growth, there was no point at which the start of the increase could be pinpointed. The dynamic range over which the response from normal to essentially no growth occurred was 10 to 20 fold (i.e. 5×10^{-5}M to 1×10^{-3}M Ni).

The change in cell morphology which occurred during growth in Ni containing medium was dependent upon both the inoculum size and the concentration of Ni in the medium (Table 16). With a 10 ml inoculum the peak cell size again occurred at 4×10^{-4}M Ni but the size was larger, 7 μm, reached after 12 h. At the 0.1 ml inoculum size the peak of cell size

Fig. 11. Effect of inoculum size on the doubling time (DT) of A. marinus cells grown at 25°C in shaken 2216E LN broth with various Ni concentrations.

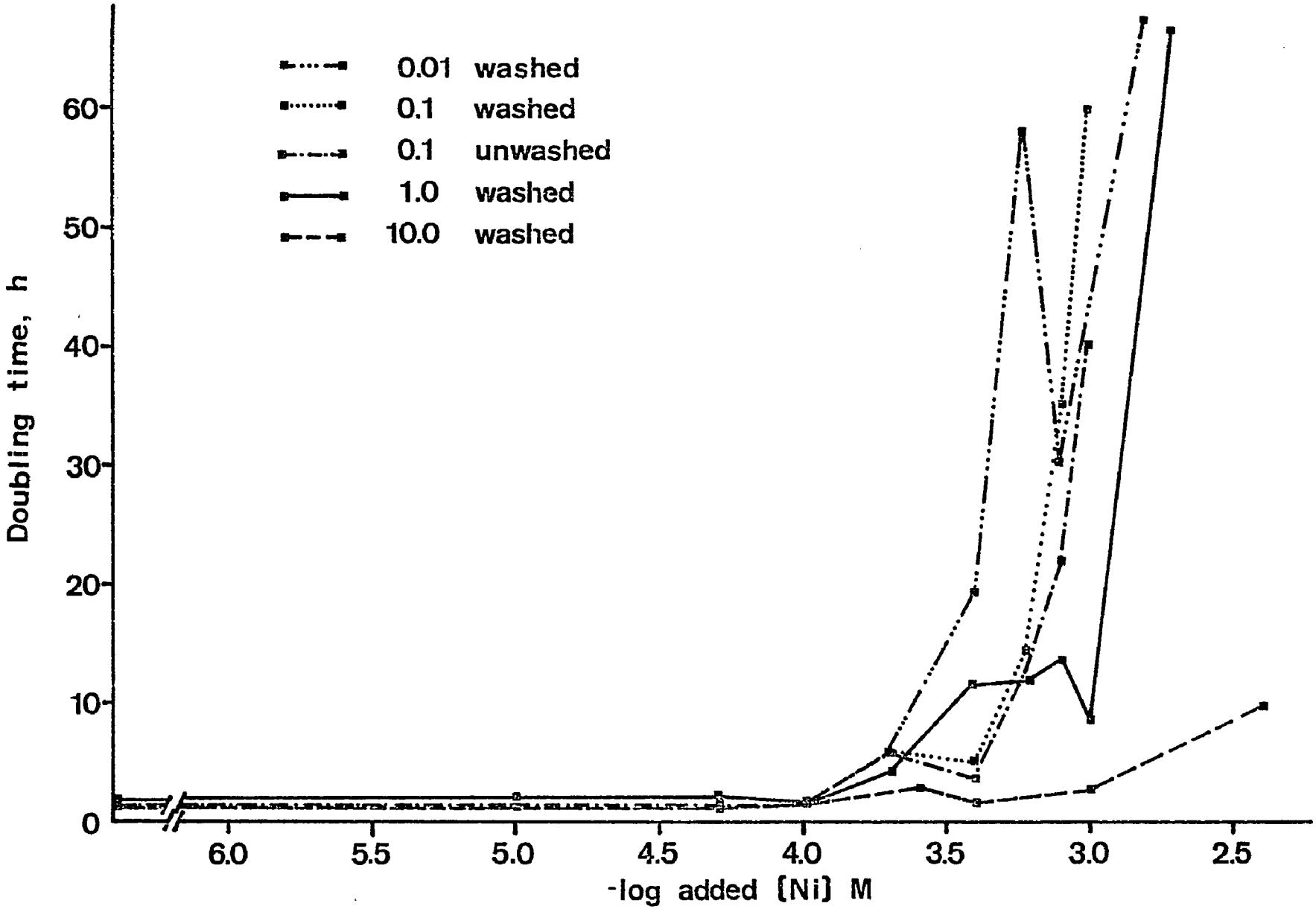

0.01 washed
0.1 washed
0.1 unwashed
1.0 washed
10.0 washed
Doubling time, h
0
10
20
30
40
50
60
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
-log added [Ni] M

Fig. 12. Effect of inoculum size on the OD_{MAX} of A. marinus cells grown at 25°C in shaken 2216E LN broth with various Ni concentrations.

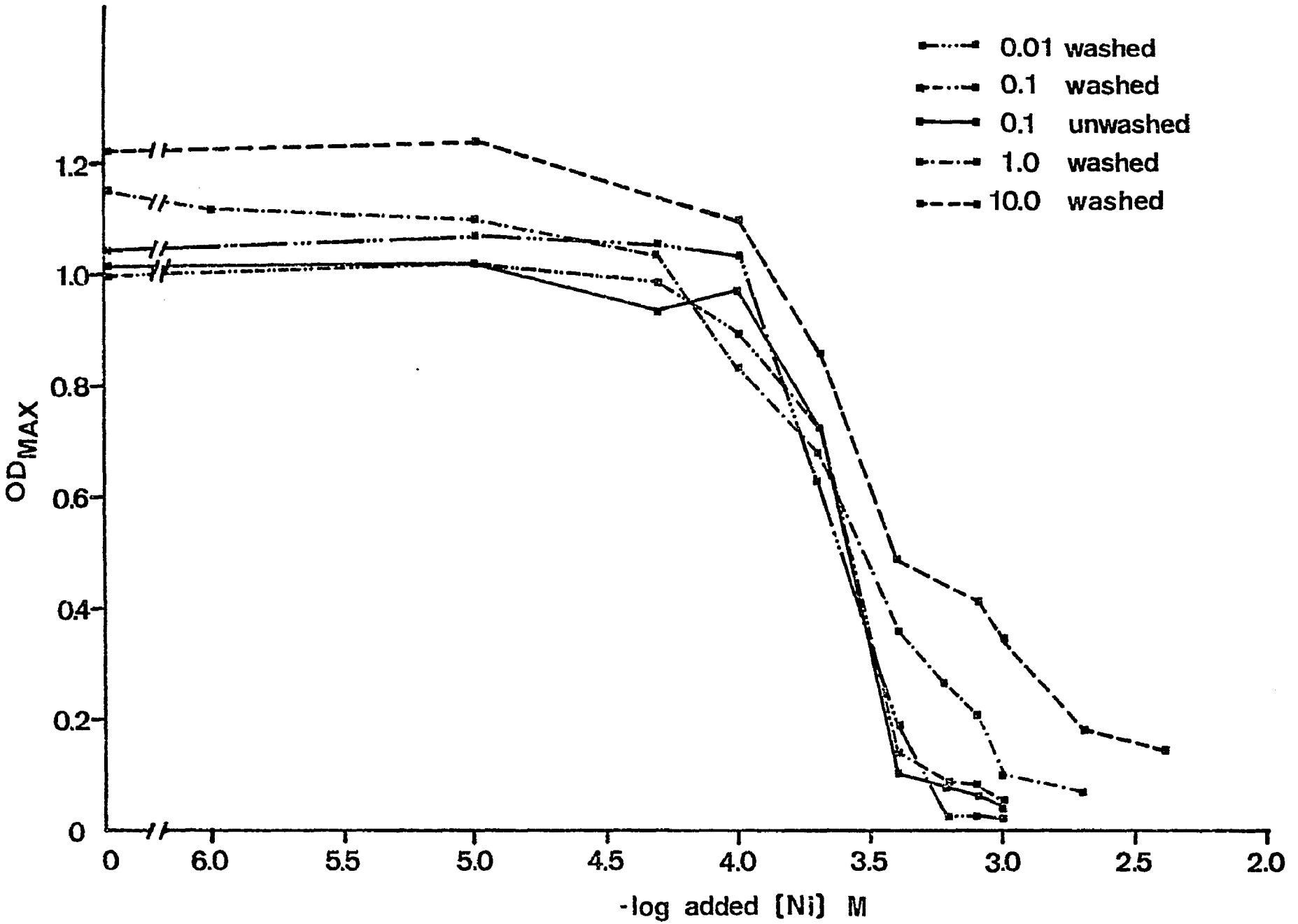

0.01 washed
0.1 washed
0.1 unwashed
1.0 washed
10.0 washed
OD MAX
1.2
1.0
0.8
0.6
0.4
0.2
0
0
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
-log added [Ni] M

Fig. 13. Effect of inoculum size on the lag time (h) of A. marinus cells grown at 25°C in shaken 2216E LN broth with various Ni concentrations.

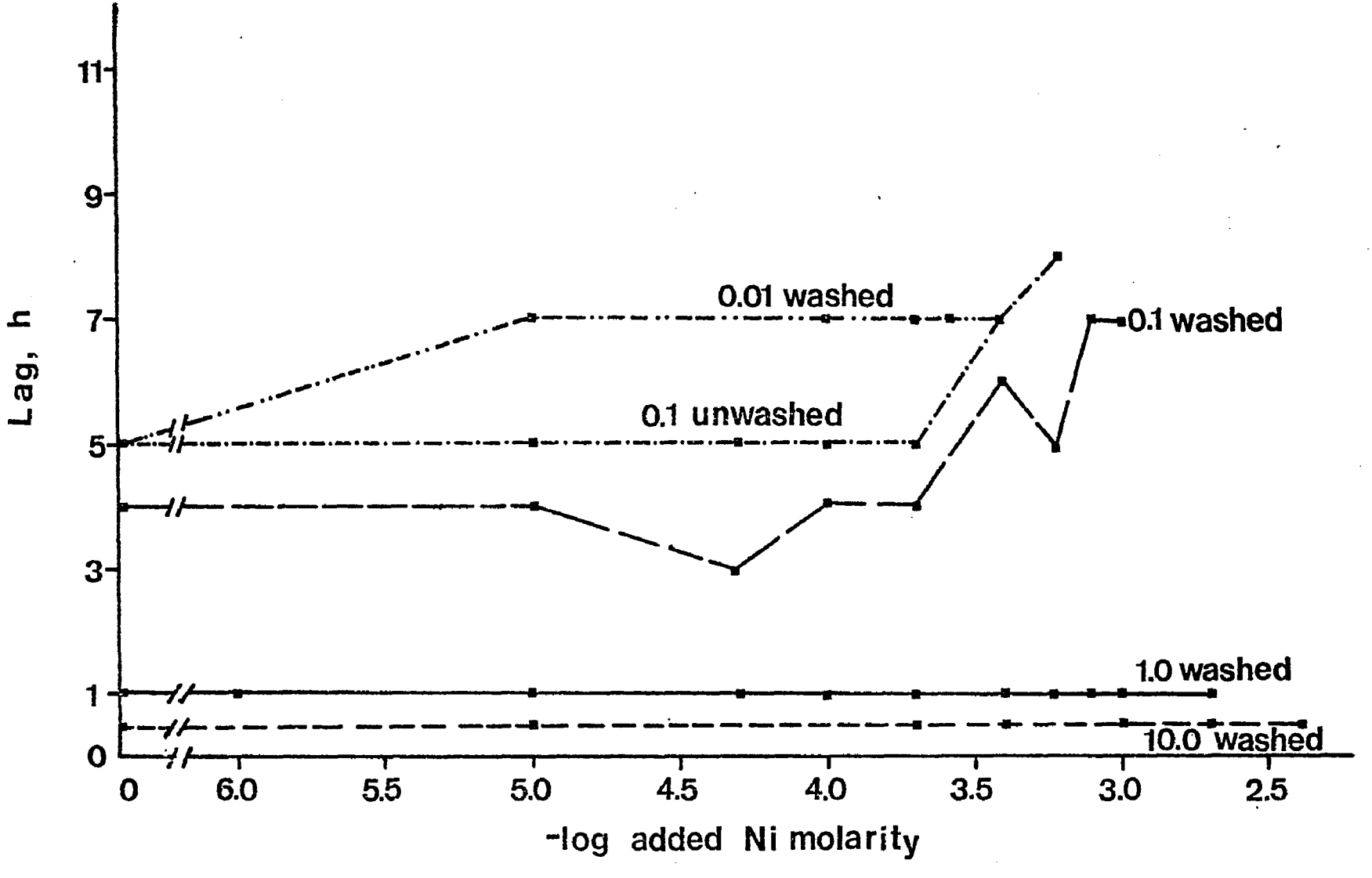

Lag, h
11
9
7
5
3
1
0
0.01 washed
0.1 washed
0.1 unwashed
1.0 washed
10.0 washed
0
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
-log added Ni molarity

Table 16. Morphological response of *A. marinus* in 2216E LN broth with various Ni concentrations and 5 inoculum treatments, recorded as maximum cell size (μm).

Ni, M	Washed 10.0 ml EQ	Washed 1.0 ml EQ	Washed 0.1 ml EQ	Unwashed 0.1 ml EQ	Washed 0.01 ml EQ
0	1 X 2	1 X 2	1 X 2	1 X 2	1 X 2
1×10^{-5}	1 X 2	1 X 2	1 X 2	1 X 2	1 X 2
5×10^{-5}		1 X 2	1.25 X 2.5	1.25 X 3	1 X 2
1×10^{-4}	1.25 X 2.5	1.5 X 3	1.5 X 4	1.5 X 3	1.5 X 3
2×10^{-4}	1.5 X 3	4	9	4 X 6	8
4×10^{-4}	4	7	12	10	12
6×10^{-4}		6	7	6	7
8×10^{-4}	4	4	5	5	5
1×10^{-3}	2	2	3	2	4
2×10^{-3}	2	2			
4×10^{-3}	1 X 2				

again was at 4 X 10^{-4}M. In this case the maximum size was reached after 10 to 12 h incubation, and the size was much greater, typically 10 to 12 μm in diameter, with individual cells observed of up to 11 X 18 μm. At the 0.01 ml inoculum size the pattern was essentially identical to that in the 0.1 ml inoculum. Thus the cell morphology was dependent on inoculum size and metal concentration, but no interaction was seen between the Ni concentration at which the morphology peak occurred (4 X 10^{-4}M Ni) and the size of the inoculum. The cell size increased up to the morphology peak and decreased as Ni rose above that point.

The formation of megalomorphic cells of A. marinus was recorded photographically. In the absence of Ni (Fig. 14) the cells in active growth (5 h) had a short, rod-shape, about 1 X 2 μm, which decreased progressively as the cells entered stationary phase (24 h), the cells becoming cocco-bacillary, about 0.75 X 1.25 μm. In 4 X 10^{-4}M Ni the cells of a 10 ml EQ inoculum (Fig. 15) progressively increased in length and width, reaching a size of about 2 X 4 μm, as pleomorphic cells with prominent vacuoles. Note that much of the increase in cell size occurred after the peak of growth (4 h; Fig. 9) had passed.

The 1.0 ml EQ inoculum size gave much larger cells than at the same 4 X 10^{-4}M Ni concentration (Fig. 16). By 4 h incubation the cells had reached as great a size as they had with the 10 ml EQ inoculum after 6 h, and they continued to increase. By 9 h the cells were 6 to 8 μm in size, roughly spherical and pleomorphic, with vacuoles comprising c. 50 %

Fig. 14. Phase contrast photomicrographs of A. marinus grown in 2216E LN broth, 1.0 ml EQ inoculum: 3 h late lag or early log phase, 5 h mid log phase, 9 h end of log phase, 24 h stationary phase.

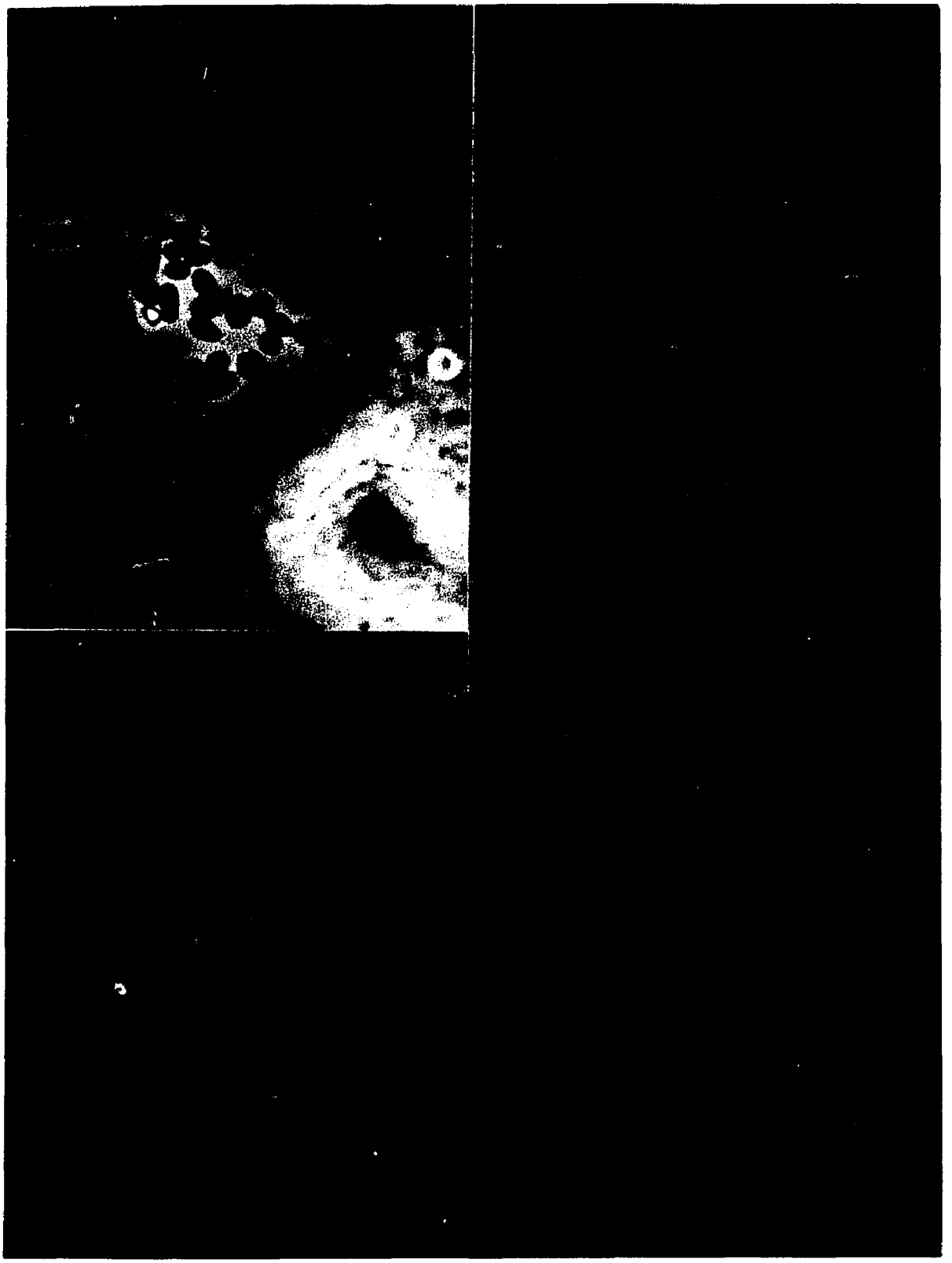
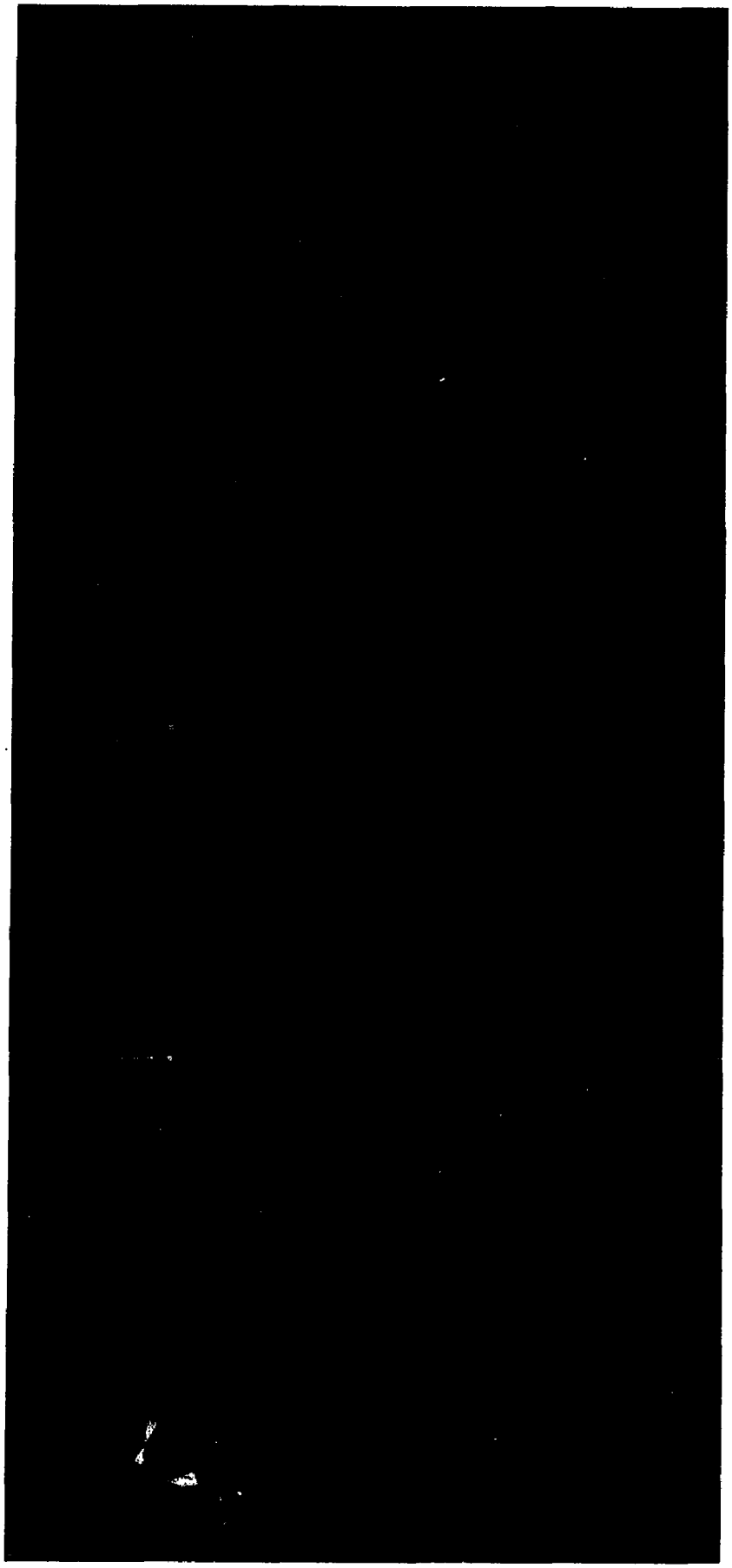

10 µm

Fig. 15. Phase contrast photomicrographs of A. marinus grown in 2216E LN broth, 4 X 10^{-4}M Ni, 10.0 ml EQ inoculum. 2 h log phase, 4 h stationary phase, 6, 8, 12 h "death" phase.


10 µm

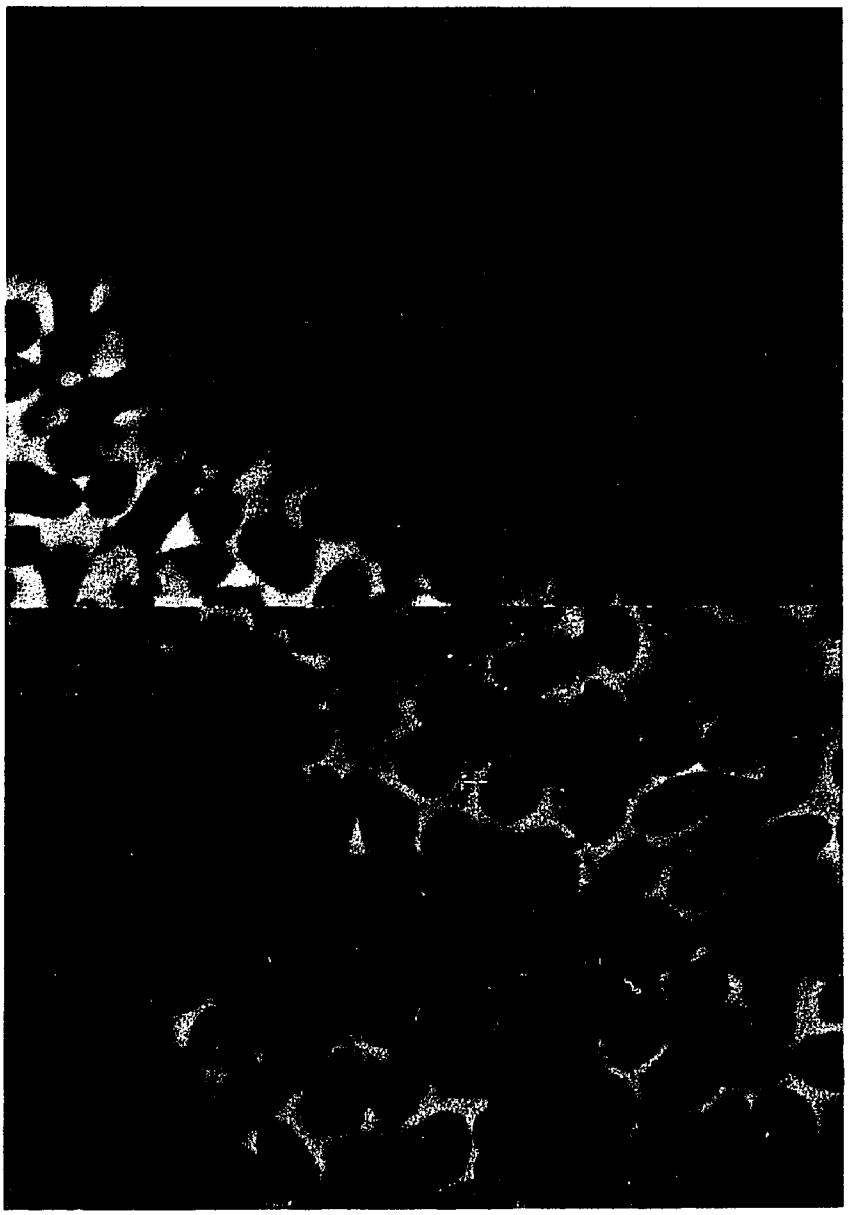

10 µm

Fig. 16. Phase contrast photomicrographs of A. marinus grown in 2216E LN broth, 4 X 10^{-4}M Ni, 1.0 ml EQ inoculum: 2-4 h lag phase, 2-12 h log phase growth, 24-48 h stationary phase.

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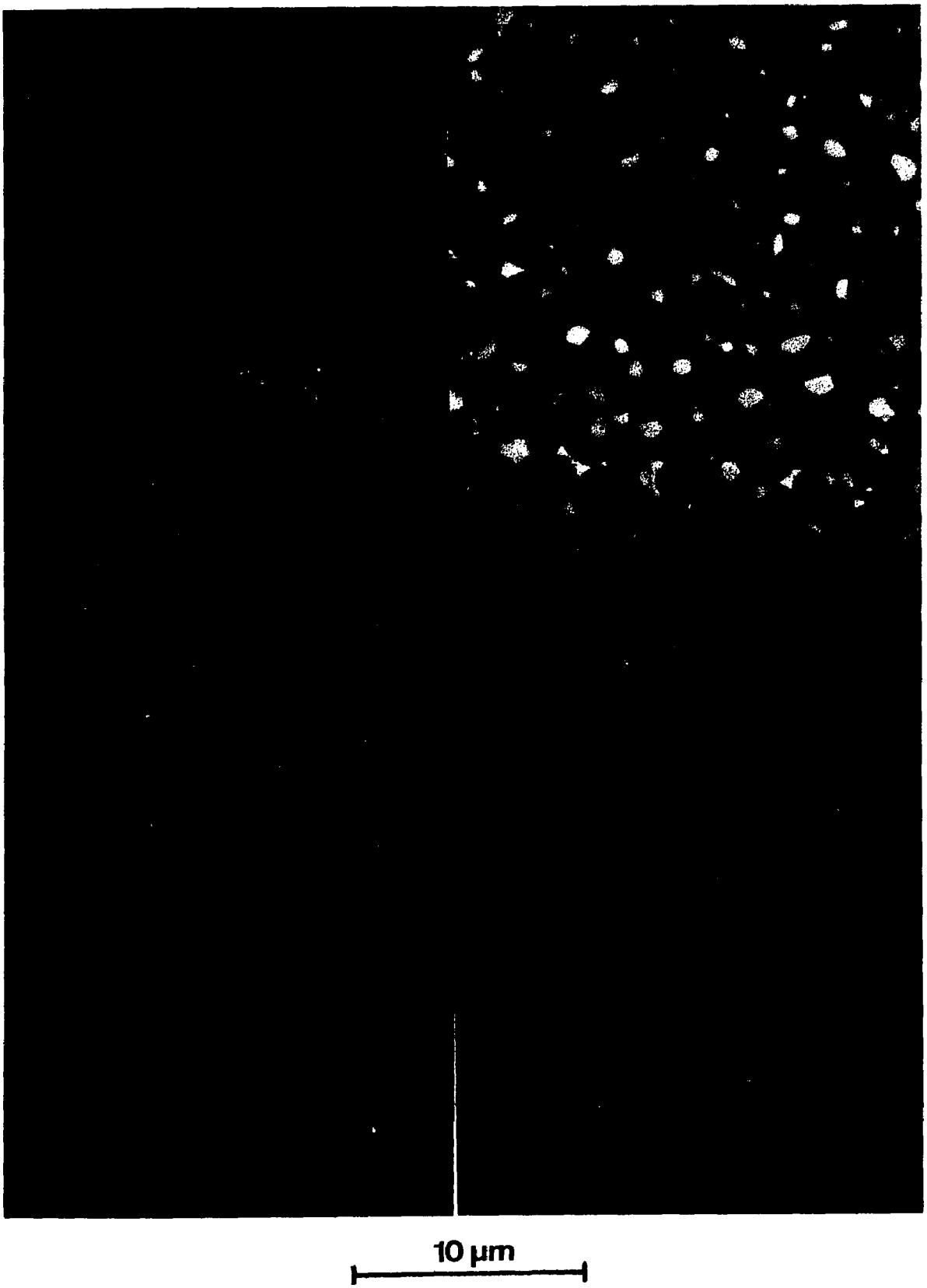
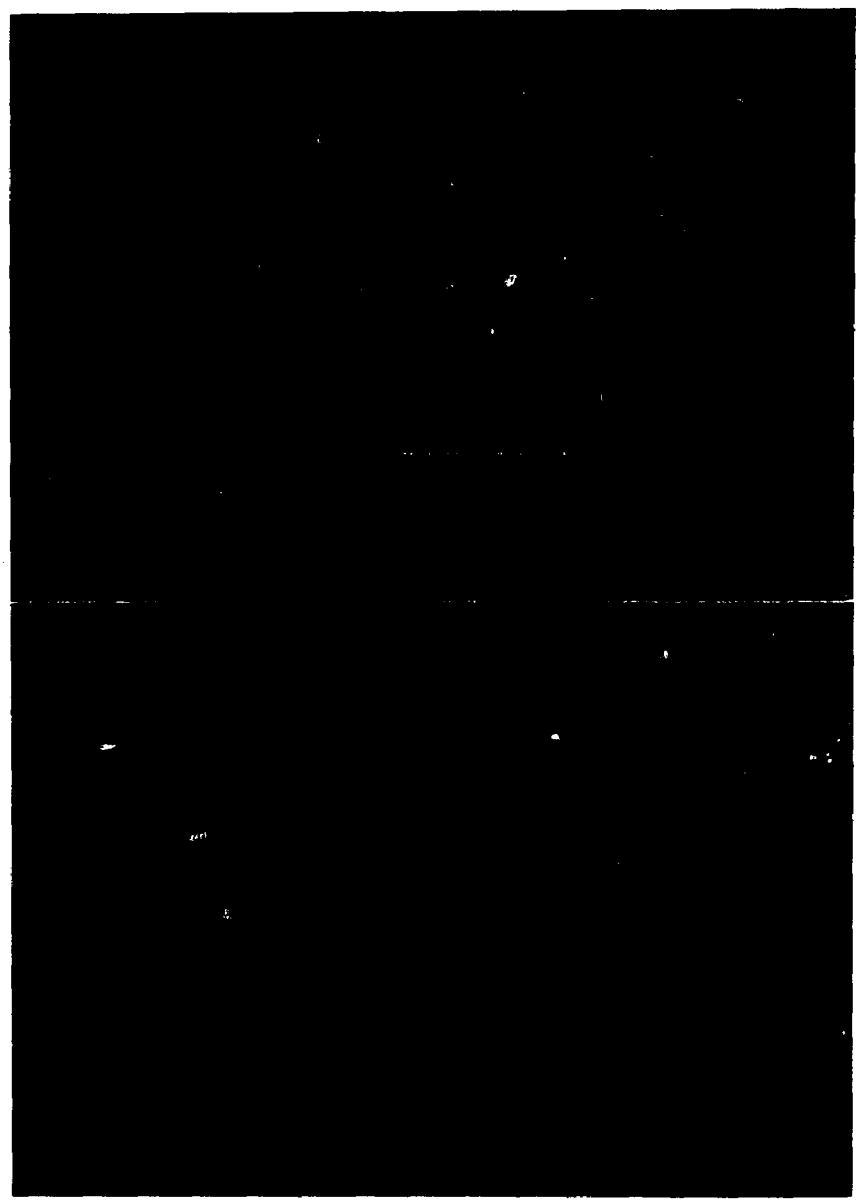
lum:

h




10 µm

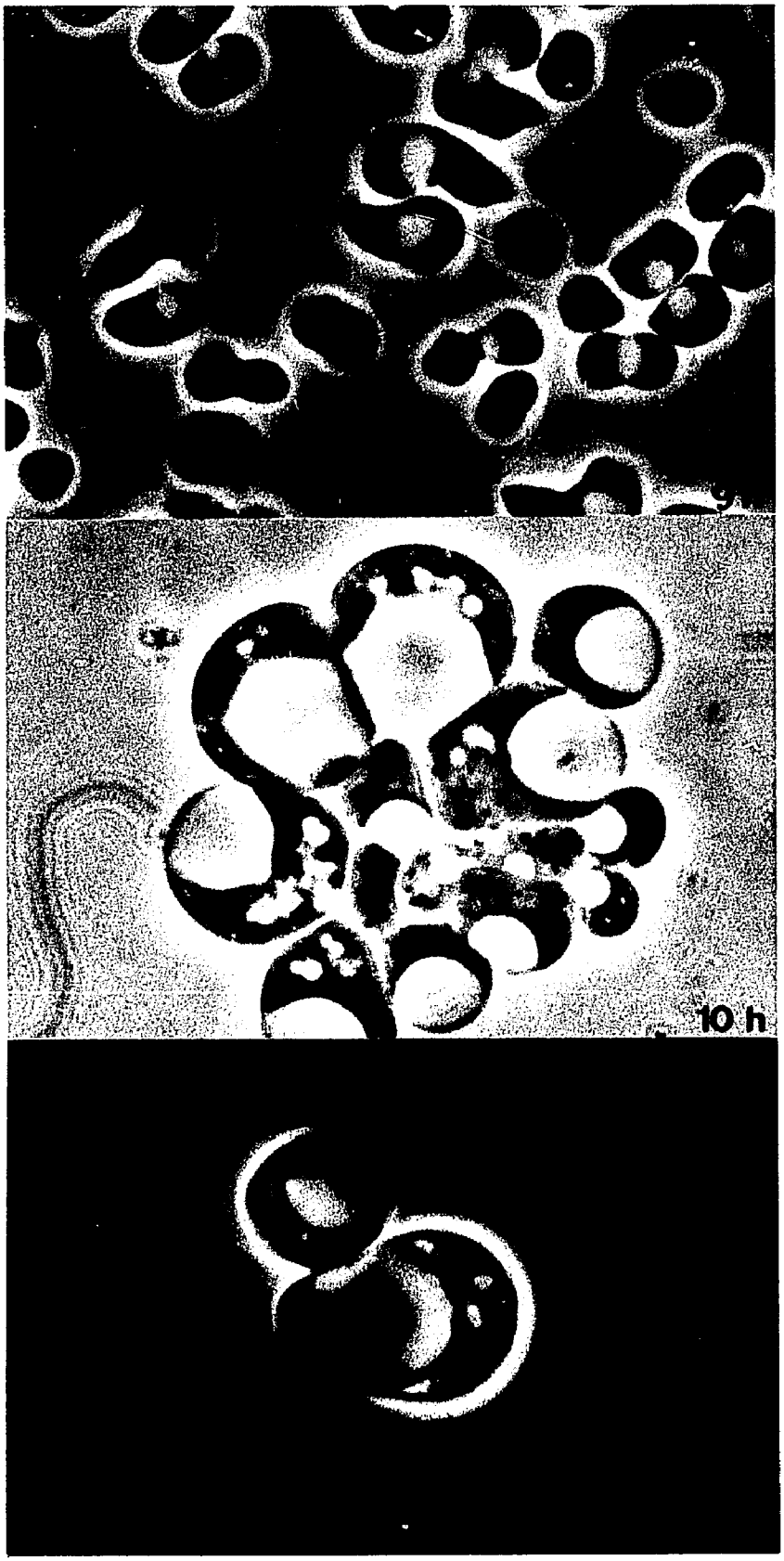
of the cell volume. By 12 h the cell size peaked at about 10 μm, and it remained essentially constant to at least 48 h. Note that some of the cells in Fig. 16, 48 h appeared to be lysing.

With the 0.1 ml EQ inoculum (Fig. 17) the pattern was similar, but the maximum cell size was greater. At 10 h incubation the cells had reached 12 μm in size, and after 24 h cells as large as 12 X 18 μm were observed. Again by 48 h incubation lysing cells and cell "ghosts" were observed. At the 0.01 ml EQ inoculum size (not shown) the pattern was similar to that of the 0.1 ml inoculum, but the onset of cell lysis and cell ghosting was faster. Note that in none of the micrographs were any normal cells seen.

In Table 16, it was noted that as the Ni concentration rose, the cell size increased to a maximum, at about 4 X 10^{-4}M Ni, with reduced cell size at greater Ni concentration. Fig. 18 illustrates this feature in 1.0 ml EQ inoculated cultures. The results of Cobet (1968) and Gonye (1972) had indicated that megalomorphic cells formed due to a decoupling of cell growth and cell division induced by critical concentrations of Ni. The cells ceased division, but continued to grow, thus forming the megalomorphs. Evidently as the Ni concentration increased above the critical 4 X 10^{-4}M concentration, inhibition of growth, as well as division, set in and the maximum cell size dropped.

These observations of the growth and morphology responses of A. marinus to Ni in a complex medium duplicated the essential features of the response as reported by Cobet (1968). His standard inoculum size (1 X 10^5 cells/ml; by CFU)

Fig. 17. Phase contrast photomicrographs of A. marinus grown in 2216E LN broth, 4 X 10^{-4}M Ni, 0.1 ml EQ inoculum: 9, 10 h late log phase, 12, 15, 48 h stationary phase. At 15 h the photomicrograph was taken with the 12.5x ocular micrometer (superimposed graduations). For this frame the μm bar subtends 20 μm.


10 h
10µm

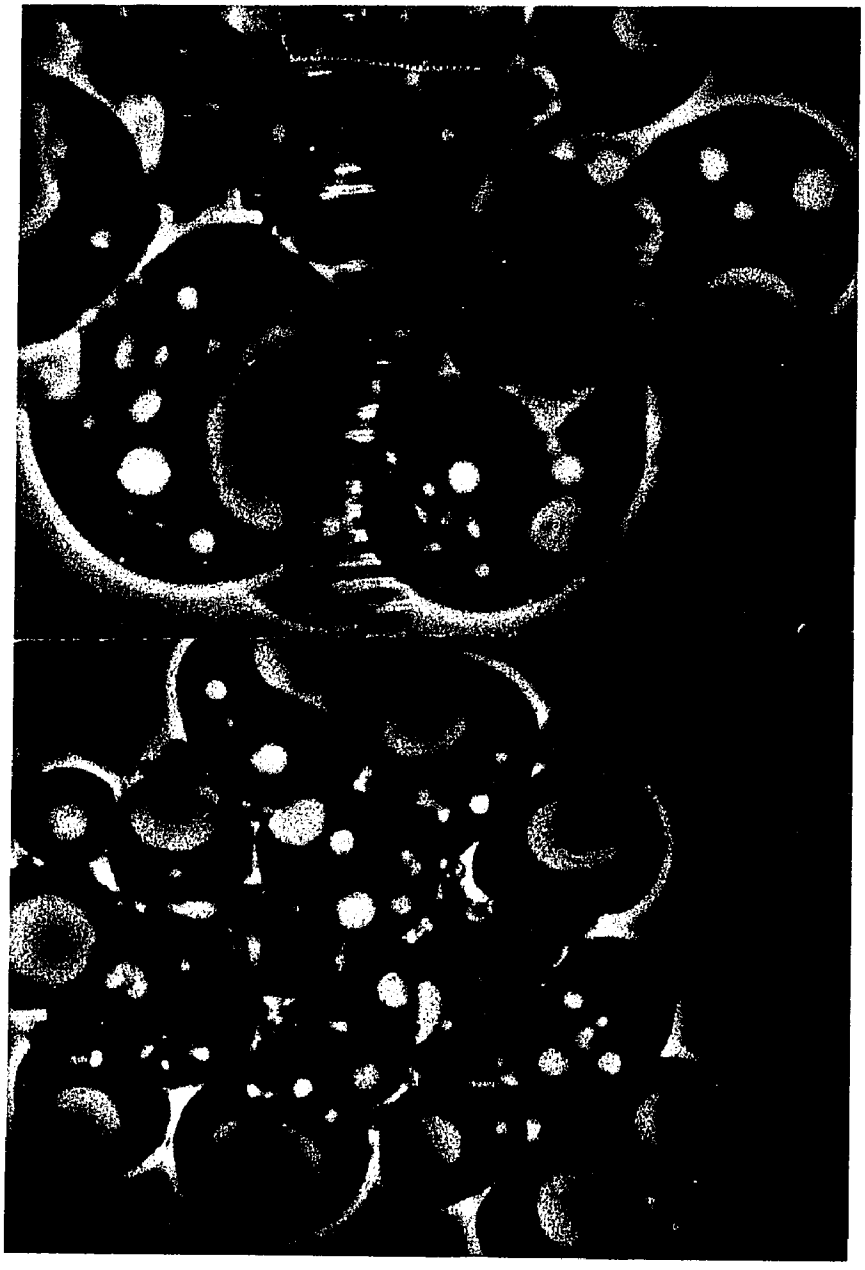
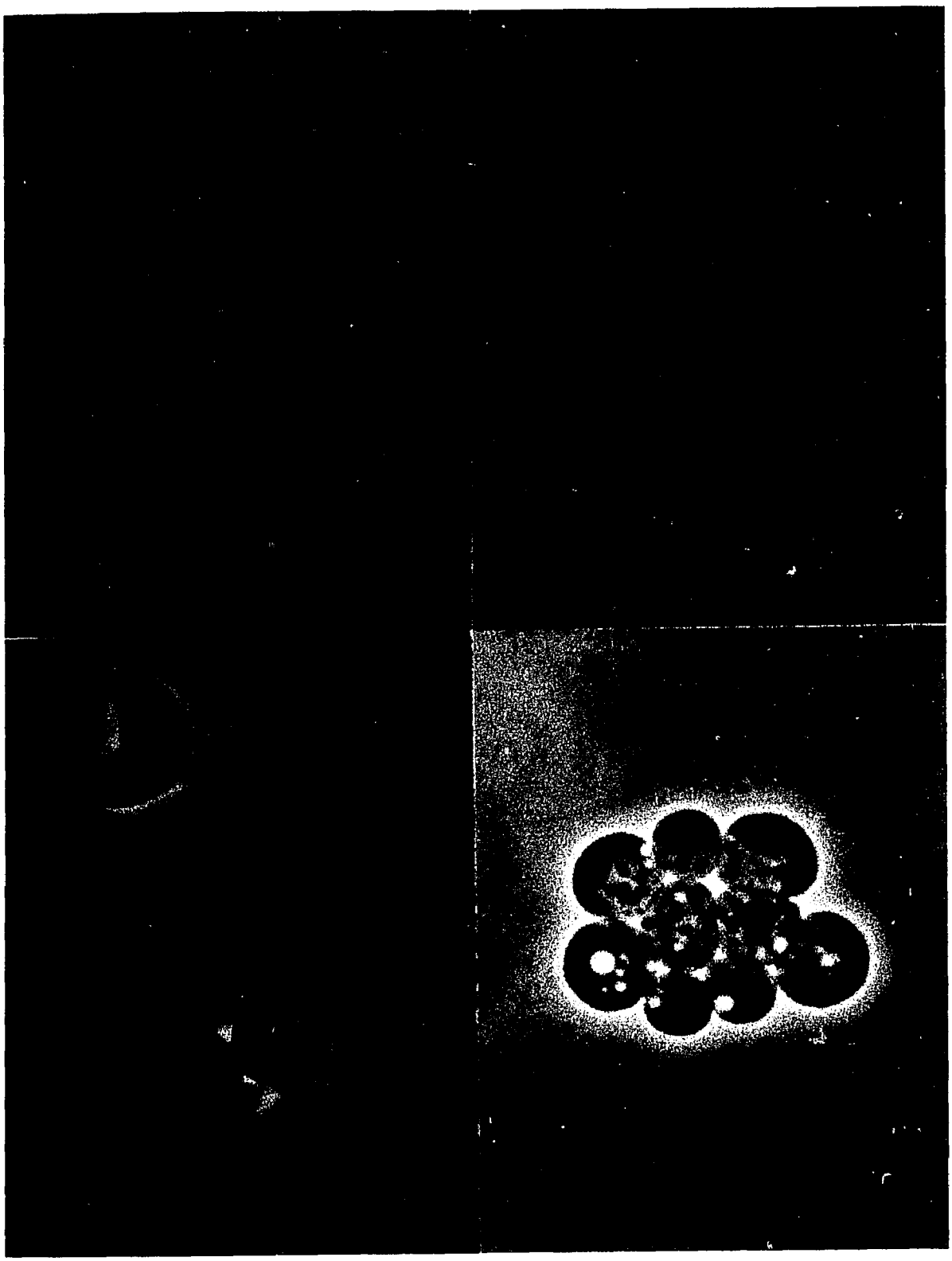
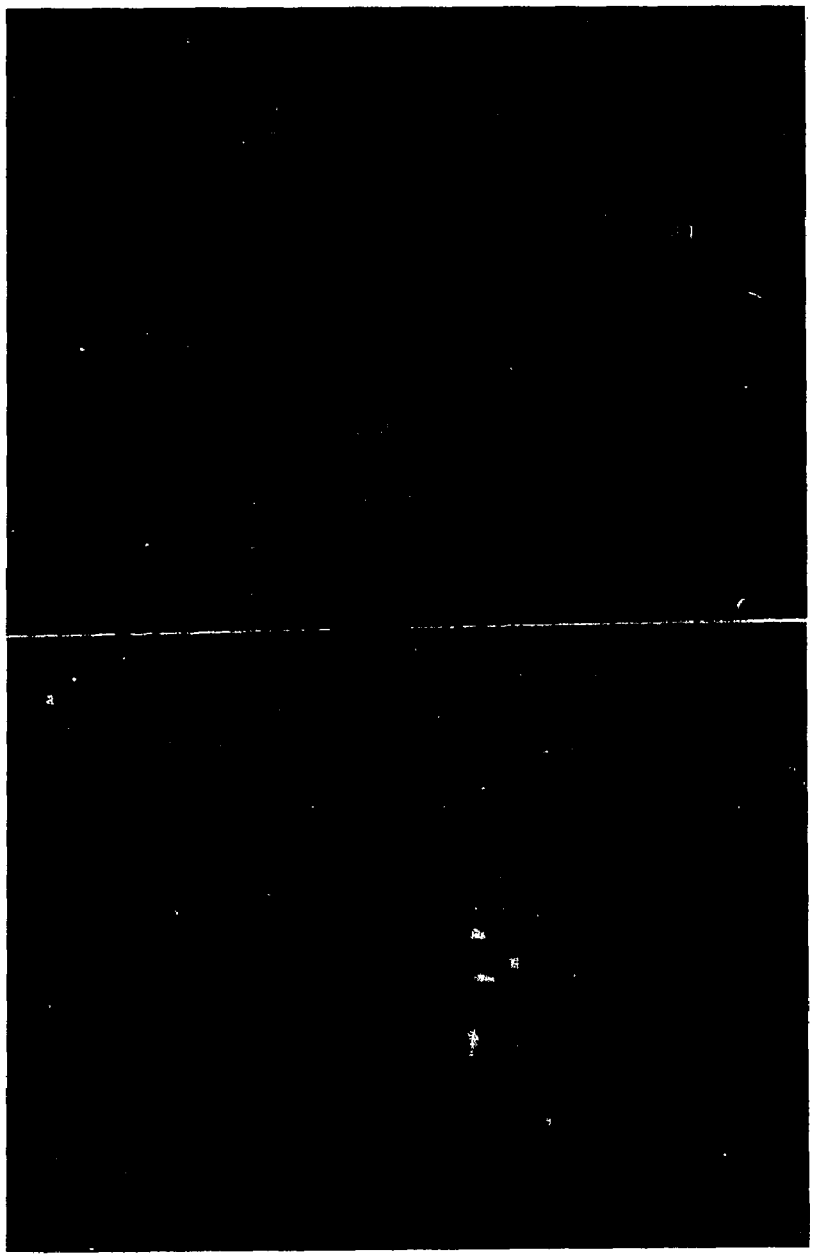

10 µm

Fig. 18. Phase contrast photomicrographs of A. marinus grown in 2216E LN broth, 1.0 ml EQ inoculum, 24 h incubation in various Ni concentrations.

own

ba-




10 µm

corresponded to 0.045 ml EQ. Comparison with the results of Gonye (1972) was more difficult as he did not specify the inoculum size used, but there also appeared to be agreement. I found the growth of A. marinus in 2216E LN to be dependent on Ni concentration, but relatively independent of inoculum size. The response to Ni in this medium was typical of a bacterial toxicant response. As the inhibitor concentration was increased above a minimal inhibitory concentration, the growth yield and rate decreased. There was little increase of culture lag due to increased inhibitor concentration. By contrast the morphology response was dependent on inoculum size, but again as with growth, the Ni concentration at which peak morphology occurred was constant, independent of inoculum size.

Slide Culture

Cobet (1968) demonstrated that cultures of megalomorphic cells were viable by showing subsequent growth of such cultures when diluted into fresh Ni-free medium after a short lag. However, the question remained whether all the megalomorphic cells were capable of growth, or whether a small (perhaps undetectable by microscopy) population of morphologically less differentiated cells was responsible for the regrowth. To answer this question the technique of slide microculture was used. A preliminary experiment (Fig. 19) demonstrated that A. marinus cells (0 Ni) showed the features of the growth of the organism in 2216E LN broth. At 2 h incubation (room temperature, c. 25°C) each of the two cells originally in the

Fig. 19. Growth of A. marinus in slide culture on 2216E LN agar, at 25°C.

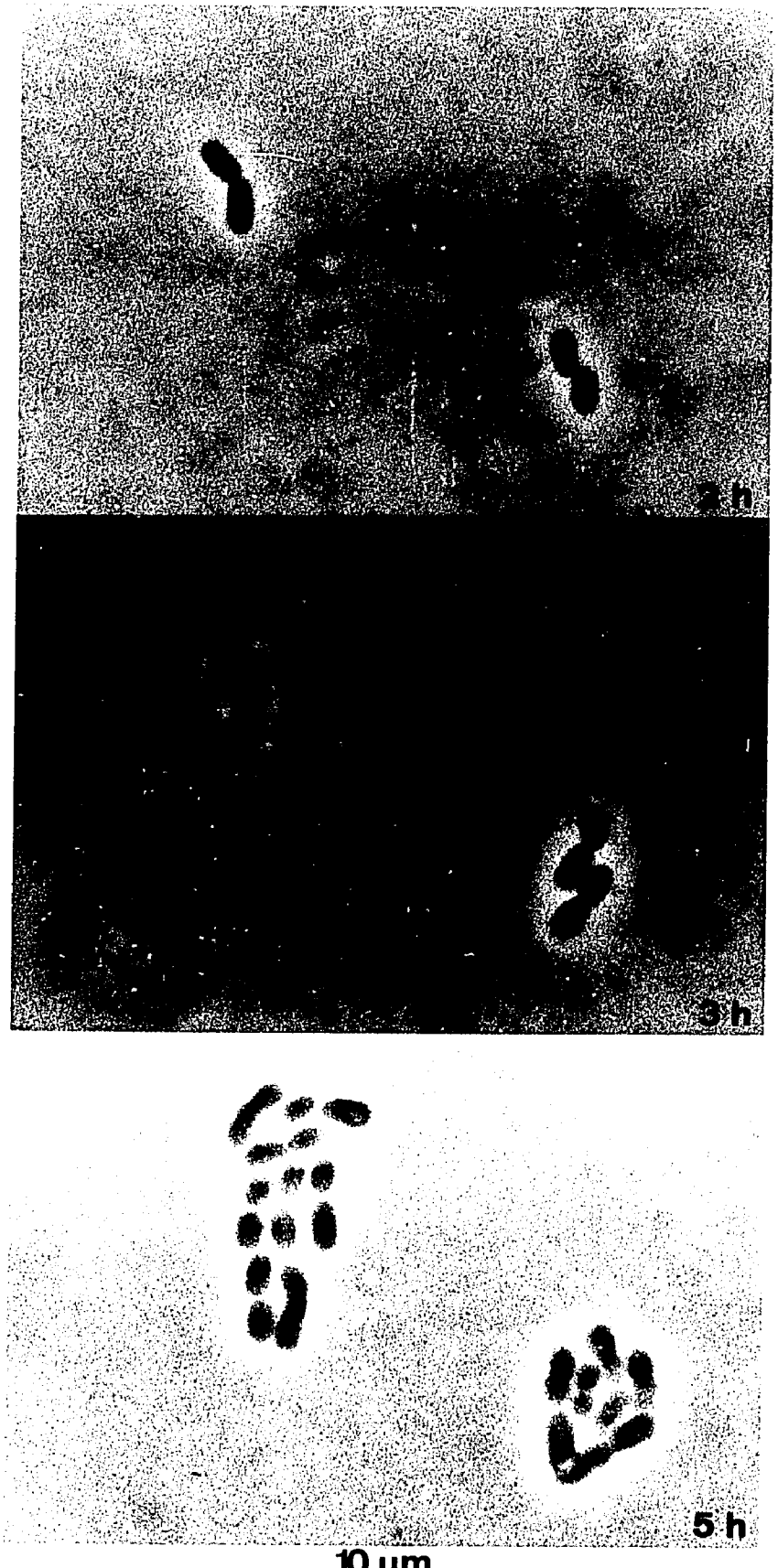

2 h
3 h
5 h
10 µm

Fig. 19 continued. At 23 h the original two colonies had broken up, these undisturbed colonies were photographed.


10 µm

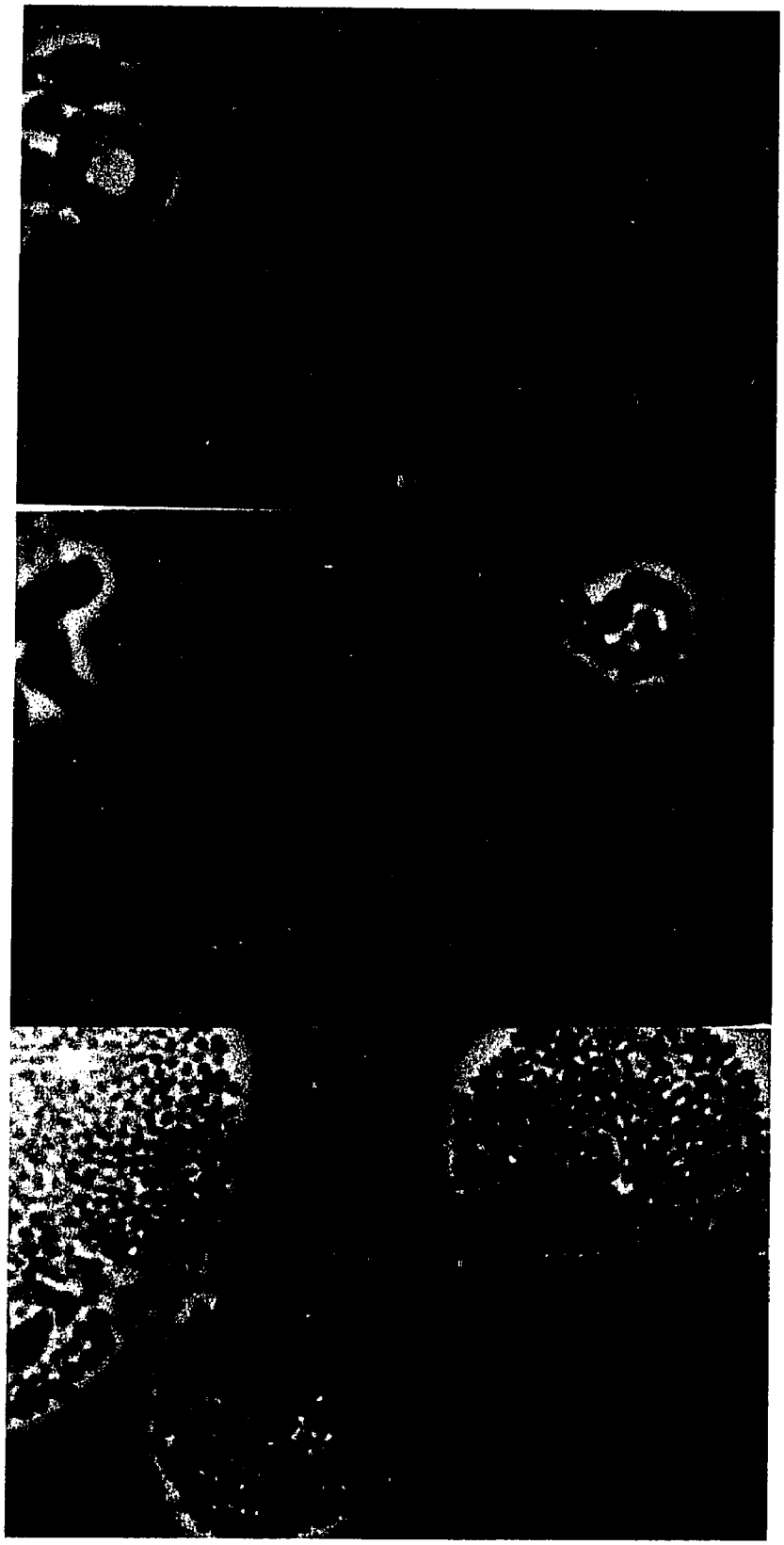
field of view had divided; at 3 h one pair had again divided giving 4 cells, the other pair had elongated and was about to divide; at 5 h the lower right cluster of cells showed at least 10 recognizable cells while the upper left cluster showed 16 cells. On the basis of the upper left cluster the doubling time of the culture was 1.0 h in excellent agreement with the growth data reported above. In the next frame shown (Fig. 19d), 8 h incubation, the original two colonies had grown to c. 60 cells, and the cell size was beginning to diminish. The last frame shows three other microcolonies at 23 h (one of the original colonies had broken up). There had evidently been no growth since 8 h, thus the cells were in stationary phase. Note the extent to which the cells had become cocco-bacillary in form. The cells were not, however, cocci.

Having thus demonstrated the ability of A. marinus to show its normal pattern of growth and differentiation in this system, I undertook to examine the ability of megalomorphic cells to revert to normal morphology when placed onto medium without Ni. A 24 h 2216E LN broth culture with 6 X 10^{-4}M Ni was used to inoculate a slide culture. The results are presented in Fig. 20. It is evident that the megalomorphic cells initially present reverted to normal morphology by a process of multiple divisions, each division giving smaller size cells until normal morphology was restored.

Uptake of Ni

Previous work by Cobet (1968) had indicated significant uptake of Ni by A. marinus grown in 2216E LN, and Jones, Royle,

Fig. 20. Reversion of megalomorphic cells of A. marinus on Ni-free slide culture. A 24 h culture of 6 X 10^{-4}M Ni 2216E LN broth grown cells were used as inoculum. The largest cell in the time 0 field (upper left) was about 8 μm in diameter. At 6 h incubation (center) the 3 μm cell present at the bottom center divided into four 1.5 μm diameter cells (DT=3 h). The large megalomorph initially present at the upper left divided into two large cells c. 2 X 5 μm in size. At 17 h (bottom) the cells were in normal stationary phase morphology.


10 µm

and Murray (1976) had found very high accumulation of Zn under the same conditions. In both studies the maximal uptake occurred at Ni or Zn concentrations just below the maximum metal tolerated by the organism. However, the direct quantitation of the trace metal composition of bacteria is difficult and suffers from poor precision (Passman, 1977). Ni uptake by A. marinus was assayed by the use of ^{63}Ni as a sensitive tracer. The isotope was added as $^{63}NiCl_2$, the same form as was added cold Ni, thus the atoms of the isotope were assumed to behave identically with the mass of Ni.

Two 100 ml volumes of 2216E LN broth with 4 X 10^{-4}M Ni and 4.16 µCi ^{63}Ni (9.23 X 10^5 DPM/ml) added were inoculated with 0.1 ml EQ of A. marinus and incubated 24 h (early stationary phase) at 25°C with shaking. Each flask was divided into two 50 ml aliquants, filtered through Millipore 0.45 µm filters, and the filters washed with two 10 ml volumes of 75 % L&FASW. The filters and blank filters washed with 75 % L&FASW were dissolved in scintillation fluid and the ^{63}Ni activity counted by LSC. The counts on the filters (50 to 60 CPM) were less than twice the blank (32 CPM). This corresponded to a maximum real uptake of 50 DPM per filter, or 1 DPM per ml of culture. This represented the uptake into cells of less than 0.001 % of the medium content of ^{63}Ni. Repeated attempts to quantitate ^{63}Ni uptake by A. marinus in 2216E LN yielded results below the limits of detection. The conclusion was unavoidable that extremely little uptake of Ni by A. marinus in this system occurred. As will be clear later this lack of uptake must strongly influence the interpretation of the

interaction of Ni with A. marinus. The disagreement of my results with those of Cobet (1968) may have been due to his use of centrifugation for the harvesting of cells, which may have led to the inclusion of inorganic precipitates into the apparent uptake. The results of Jones, Royle, and Murray (1976) using Zn^{++} while doubtless having quantitated a real uptake, were studying a metal other than Ni, which may be translocated by a separate system.

Electrochemical Analysis

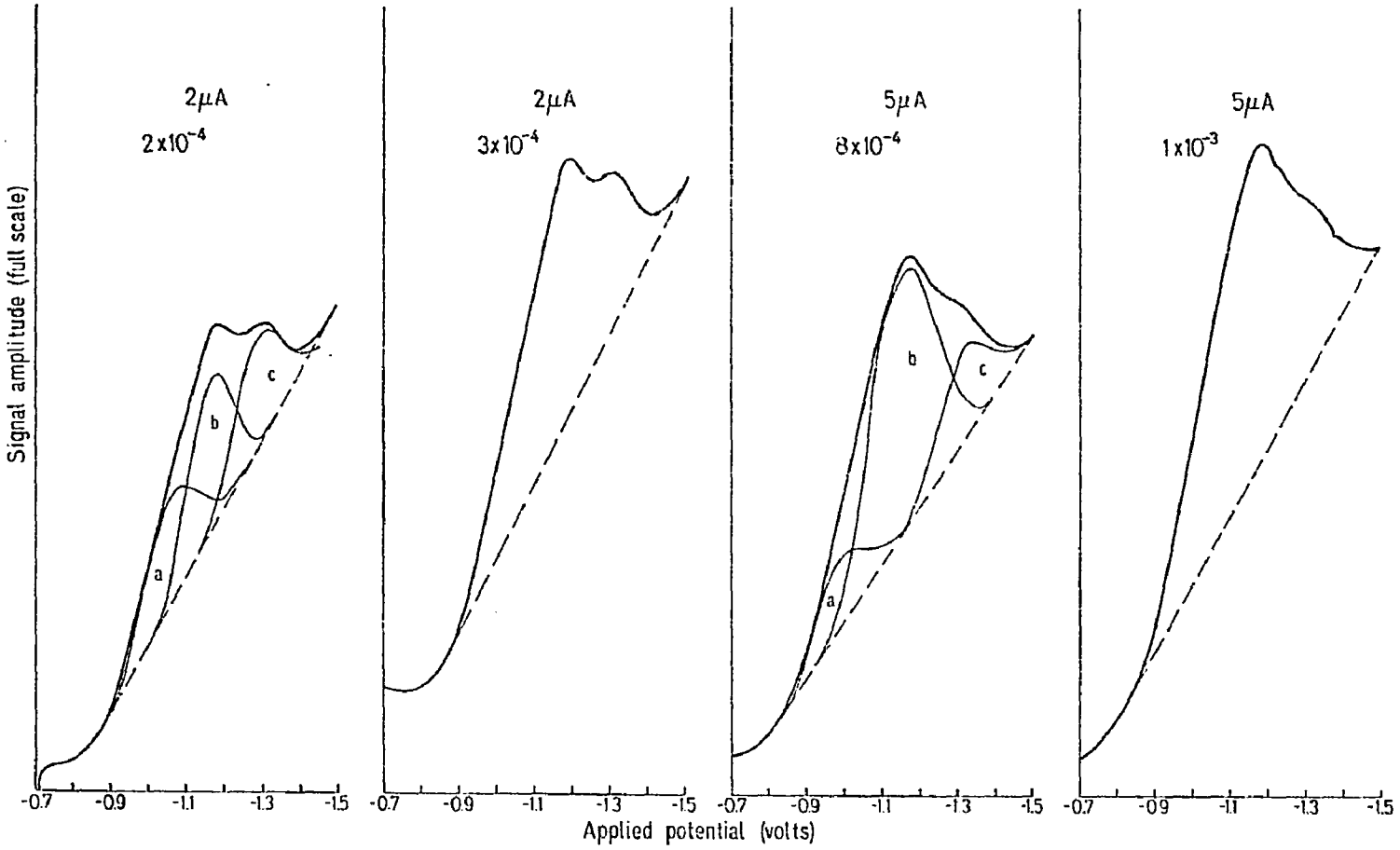
The previous workers with the A. marinus - Ni - 2216E LN system had proposed that the point at which Ni toxicity effects first appeared (c. 1 X 10^{-4}M Ni) was the point at which the added Ni exceeded the metal-binding capacity of the organic constituents of the medium, and free metal ion appeared (Cobet, 1968). The complexity, and undefined nature, of the peptone yeast extract medium made direct estimation of its metal-binding capacity impossible. Differential pulse polarography is a metal analysis technique which will detect the occurrence of complexed forms of metal ions in solution. It was used with 2216E LN medium to answer two questions: 1) could the point at which free Ni ion appeared in the medium be detected? and 2) did the speciation of Ni in 2216E LN change during the growth of A. marinus?

In an effort to answer the first question an experiment was set up in which batches of media were prepared having total organic nutrient (peptone and yeast extract in a 1:1 ratio) concentrations graded from 0 to 2 g/liter in 75 % L&FASW. These

were divided into 100 ml aliquots, and graded concentrations of Ni from 0 to 1 X 10^{-2}M added. The complete set of 64 flasks (8 nutrient concentrations X 8 Ni concentrations) was autoclaved and each polarographed. The polarograms were curve resolved to determine the speciation of Ni in the solutions, and the point in the range of increasing Ni concentrations in each nutrient concentration at which the available complexing ligands became saturated by Ni and free metal ion appeared. Typical polarograms of 2216E LN (1.0 g/liter nutrients) are shown in Fig. 21. Curve resolution determined as many as six contributing peaks in such a trace, several of which were not visible to the naked eye. Three component peaks are indicated in two of the Fig. 21 polarograms. The steeply sloping baseline on which the Ni peaks appear was due to an off-scale peak due to an electroactive component of the nutrients.

In the absence of added organic nutrients the peak due to Ni (as free ion or weak inorganic complexes) occurred at c. -1.09 v. Analysis of the polarograms of samples containing organic nutrients demonstrated that while a complex of peaks occurred in that potential range (Fig. 21), it was not possible to identify any one peak as free Ni, even at Ni concentrations (i.e. 1 X 10^{-3}M Ni; Fig. 21) at which abundant free Ni had been presumed to be present. At the present status of our understanding of the speciation of Ni in 2216E type media, we can only state that there are multiple Ni reduction peaks occurring in the region characteristic of Ni of which one may be free ion.

Fig. 21. DPP plots of 2216E LN broth with Ni concentrations of 2, 3, and 8 X 10^{-4}M, and 1 X 10^{-3}M, conducted at a sensitivity of 2 μA full scale or 5 μA sensitivity (y axis) as indicated. The x axis is in units of potential in volts versus a saturated calomel reference electrode (sce). The straight line across the base of each curve represents an approximation of the true baseline. Plots of 2 and 8 X 10^{-4}M Ni indicate the approximate contributions of 3 constituents labeled a, b, and c which give the original curve envelope when summed.


2μA
2x10⁻⁴
2μA
3x10⁻⁴
5μA
8x10⁻⁴
5μA
1x10⁻³
a
b
c
Signal amplitude (full scale)
-0.7
-0.9
-1.1
-1.3
-1.5
Applied potential (volts)

I next attempted to ascertain whether there were detectable changes of the polarographic pattern of Ni in 2216E LN medium, during growth of A. marinus, as by the production or consumption of organometallic complexes. Two sets of 36 flasks were prepared, each from a separate batch of 2216E LN broth prepared in 75 % L&FASW. One batch was prepared with 2 X 10^{-4}M Ni, the other with 4 X 10^{-4}M Ni. Eighteen flasks of each batch were inoculated with 0.1 ml EQ of a washed inoculum; the other 18 flasks were kept as uninoculated controls. All flasks were incubated at 25°C with shaking. At intervals a pair of flasks, one inoculated and one control, were removed, the OD of the inoculated culture determined to monitor growth and a sample polarographed. At each sampling time the inoculated polarogram was compared to the control in order to compensate for any drift in the instrument.

The growth data for these two experiments are presented in Fig. 22. As expected the 2 X 10^{-4}M Ni concentration showed considerable growth, while the increase of OD of the 4 X 10^{-4}M concentration was much less. The 2 X 10^{-4}M culture had a DT of 2.07, an OD_{MAX} of 0.73 and a lag of 5 h. The 4 X 10^{-4}M culture showed a DT of 7.35, an OD_{MAX} of 0.23 and a lag of 3 h.

Polarograms from the two experiments are shown in Fig. 23. Both the inoculated and control curves at 10 h incubation (mid log phase) are shown. In each case there were differences apparent between the inoculated and control curves. Resolution of the polarograms produced relative magnitudes for the six components of the complex Ni peak. Those components all underwent complex patterns of increase and decrease during the

Fig. 22. Growth of A. marinus in 2216E LN broth 0.1 ml EQ inoculum, at 2 X 10^{-4}M and 4 X 10^{-4}M Ni.

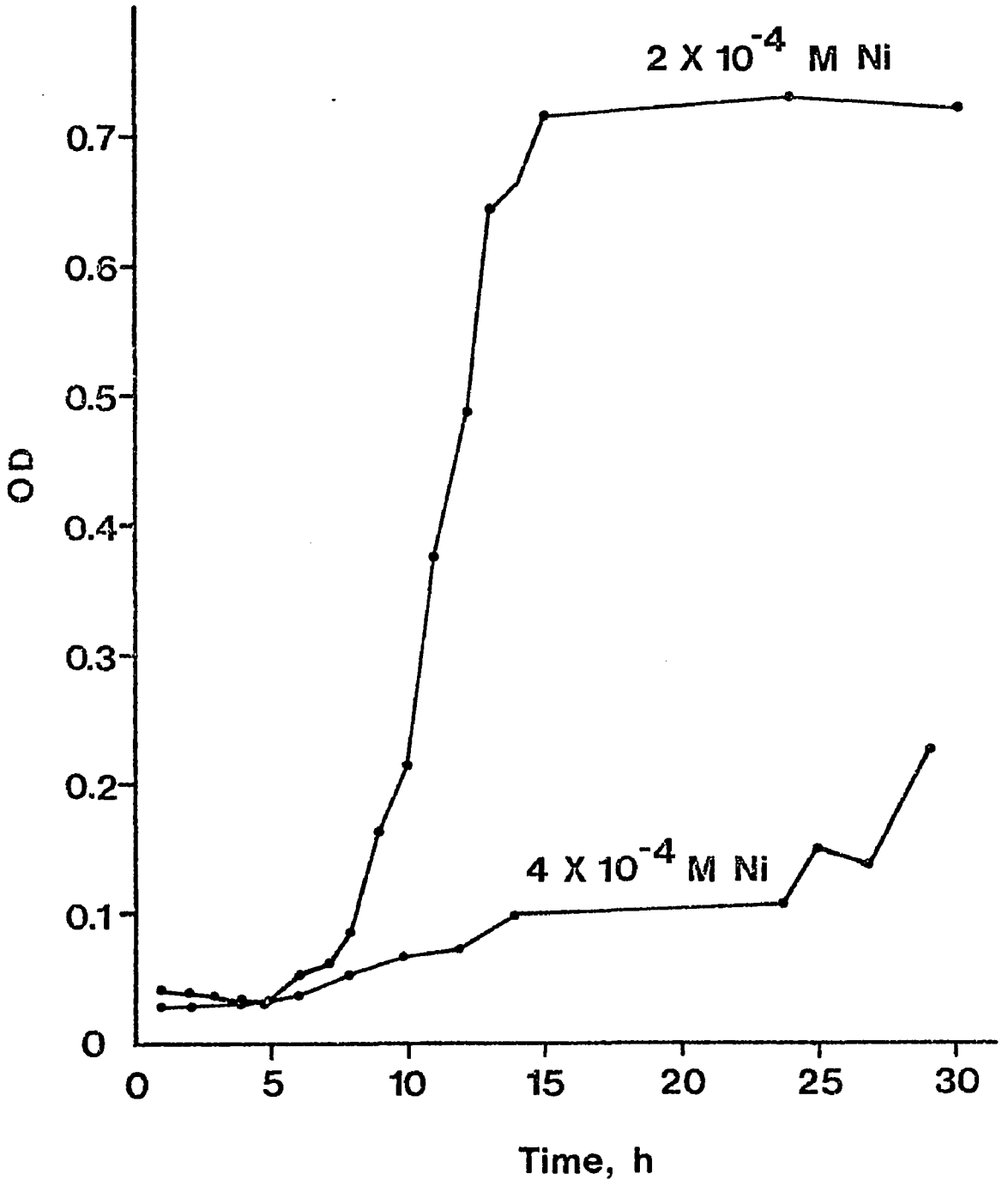
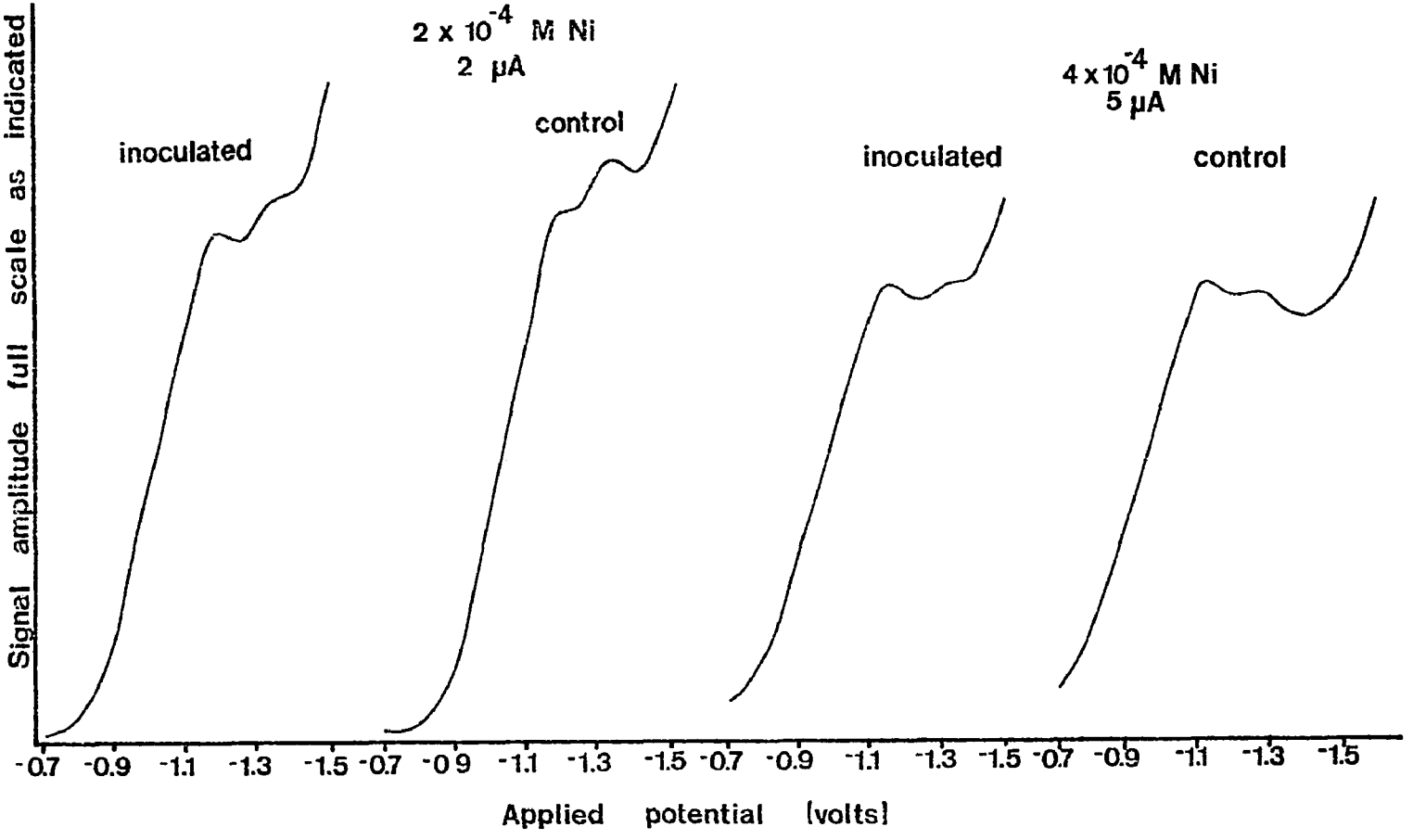

2 X 10^-4 M Ni
4 X 10^-4 M Ni
OD
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
25
30
Time, h

Fig. 23. Representative polarograms of 2216E LN broth of 2 X 10^{-4}M Ni or 4 X 10^{-4}M Ni inoculated with 0.1 ml EQ A. marinus compared to uninoculated media.

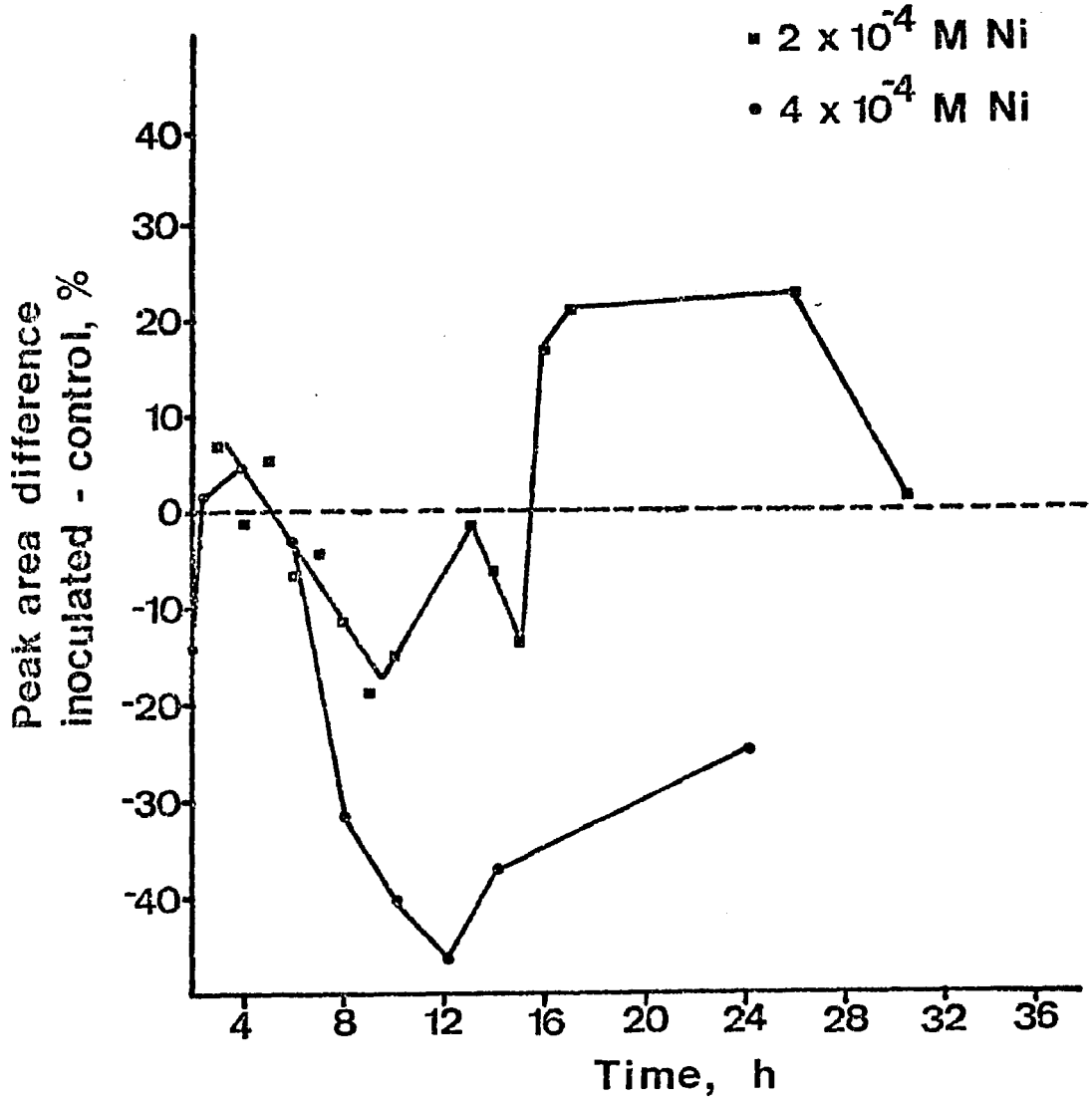

Signal amplitude full scale as indicated
2 x 10^-4 M Ni
2 µA
inoculated
control
4 x 10^-4 M Ni
5 µA
inoculated
control
-0.7 -0.9 -1.1 -1.3 -1.5 -0.7 -0.9 -1.1 -1.3 -1.5 -0.7 -0.9 -1.1 -1.3 -1.5 -0.7 -0.9 -1.1 -1.3 -1.5
Applied potential (volts)

growth of the organism. Owing to the extremely tentative nature of understanding of the chemical speciation of the components being reduced at each of the potentials, little could be inferred from that data and it has not been presented here.

At the present level of comprehension the changes which occurred in the total Ni peak were understood more readily. Fig. 24 shows the change in the total area (the area enclosed by the multiple peaks). While there were complicated changes in the size of the total peak, particularly in the 2×10^{-4}M Ni concentration after the cultures had reached stationary phase (c. 14 h), during the period of lag and log phase growth there was a consistent negative shift of the size of the inoculated Ni peak area relative to the control. Multiplying the apparent rate of Ni peak area change by the concentration of Ni present in the medium yielded "loss" rates of 7.2×10^{-6}M Ni/h for the 2×10^{-4}M cultures and 2.6×10^{-5}M Ni/h for the 4×10^{-4}M cultures. Thus, a two fold increase of the medium Ni concentration gave a 3.6 fold higher Ni peak decrease rate.

The decrease of the Ni peak area could arise from two possible mechanisms. The cells could be taking up Ni into their interiors where it would not be detected by the instrument. Alternatively, the decrease could be due to the conversion of the Ni to a form undetectable by the instrument, such as an organic complex. As the studies of ^{63}Ni uptake (above) in this medium have indicated, the accumulation of Ni by the cells of A. marinus was much less than 1 % of the Ni present in the medium.

Fig. 24. Area change of total Ni peak as percent of control (y axis) versus incubation time (h) (x axis) for (A) 2 X 10^{-4}M and (B) 4 X 10^{-4}M Ni 2216E LN broth, 0.1 ml EQ inoculum of A. marinus.


▪ 2 x 10⁻⁴ M Ni
• 4 x 10⁻⁴ M Ni
Peak area difference
inoculated - control, %
40
30
20
10
0
-10
-20
-30
-40
4
8
12
16
20
24
28
32
36
Time, h

Since the peak area decrease by the end of log growth was 25 % for the 2 X 10^{-4} cultures, and 51 % for 4 X 10^{-4}M Ni cultures, clearly far too much Ni became undetectable for cell uptake to explain it. Thus, the peak area decrease was due to the formation of electrochemically inactive complexes of Ni by the organism during its growth.

It was impossible to detect electrochemically whether free Ni ion occurred in 2216E LN medium at any Ni concentration studied, or if changes of Ni speciation with changes of Ni concentration occurred which could be correlated with the Ni toxicity response of A. marinus in 2216E LN. However, during the growth of A. marinus in 2216E LN at partially inhibitory Ni concentrations (2 and 4 X 10^{-4}M Ni) there were quantifiable decreases of the total Ni peak area which correlated with the growth of A. marinus and which corresponded to the conversion of large amounts (25 and 50 %, respectively) of the Ni initially present to electrochemically inactive forms by the end of log phase growth. This was interpreted as due to the formation of electrochemically inactive organo-metallic complexes by the organism during its growth in that medium.

Defined Composition Medium

Buffered M9

Defined composition media were used for two purposes. Firstly, a medium of known and simple composition would allow the speciation of the metal to be understood under conditions which in the complex medium response could not be further dissected. Secondly, the proper choice of constituents would

allow a medium in which the initial concentrations of metal complexation was low. As A. marinus was capable of growth on glucose as a sole source of carbon and on ammonium as a sole source of nitrogen, basal M9 medium was composed of 75 % ASW with glucose, NH_4Cl, and K_2HPO_4. Owing to the strong production of acidic end-products from carbohydrates, this medium initially was prepared with a pH buffer added to control the pH drop in the medium. The first buffer used was tris-(hydroxymethyl)-aminomethane (TRIS). This compound is frequently used as a biological buffer, and at least one reference (Gueffroy, 1975) lists this buffer as having "negligible" binding constants for metals including Cu.

The TRIS-M9 medium was used with the assumption that the components of the medium would give much less complexation than 2216E LN did. On this basis, it was expected that the concentration of Ni at which toxicity to A. marinus occurred would be less, perhaps much less, than the toxic concentration in 2216E LN medium. It was also proposed that the use of this medium might allow the definition of an intrinsic Ni toxicity concentration in A. marinus, that is the concentration of Ni that was toxic in the absence of metal complexing agents.

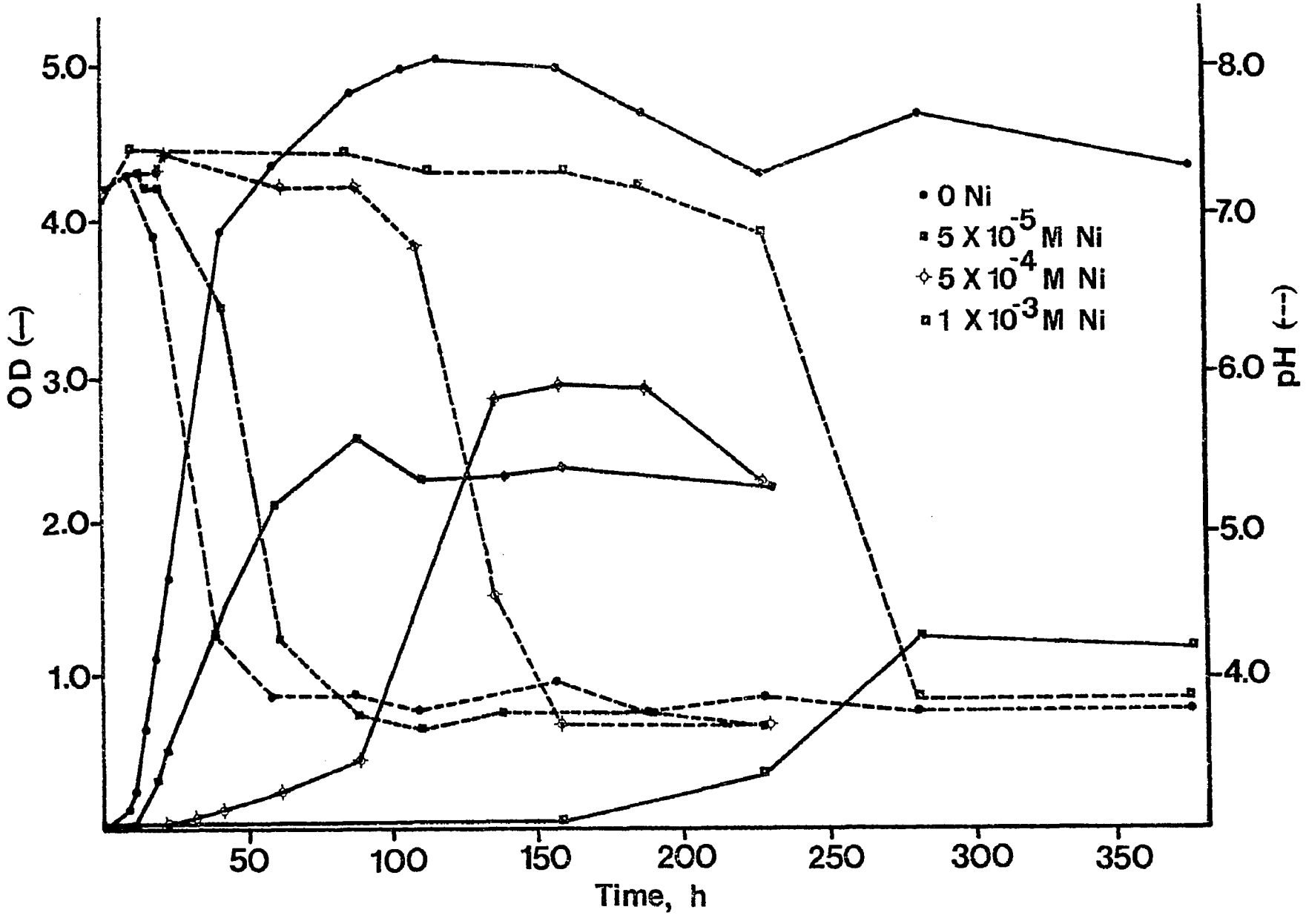
Growth studies were undertaken in this medium at a wide range of added Ni concentrations to determine that range of Ni concentrations over which the toxicity response of A. marinus occurred. Ten 100-ml flasks of TRIS-M9 were prepared with added Ni concentrations (molar) of 0, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 5×10^{-3}. Each flask was inoculated with 1.0 ml EQ of an

unwashed starter, and the OD and pH monitored at intervals during incubation with shaking at 25°C. the results for four selected Ni concentrations are presented in Fig. 25. The growth response in TRIS-M9 without Ni added showed a typical bacterial growth pattern in which an initial period of rising growth rate was followed by a period of constant rate logarithmic growth which gradually leveled off into a stationary phase. The magnitude of the peak OD attained (c. 5) was much greater than the peak OD in 2216E LN medium (c. 1.2). Filtration of a stationary phase TRIS-M9 culture through membranes of varying porosity showed that greater than 99 % of the turbidigenic material had an effective size greater than 0.8 μm. The TRIS-M9 medium contained more substrate (5 g/liter) than 2216E LN (1 g/liter) which perhaps accounted for part of the difference in growth yield. It was also likely that the three-fold difference in the dry weight:OD relationship, which was noted above in the Measurement of Growth section, accounted for a part of the difference.

There was also a sharp drop in the culture pH during the growth of A. marinus which followed a pattern essentially inverse to the growth curve (Fig. 25). There was some increase of OD (from 4 to 5) in the 0 Ni culture even after the pH had fallen below 4 confirming the pH range reported in the Taxonomy section.

At 5×10^{-5}M Ni the culture showed some manifestation of Ni toxicity, expressed as a reduced growth rate, growth to a lower OD_{MAX} and initiation of growth only after an appreciable lag time (88 h). As in the control, the pH drop mirrored the

Fig. 25. Growth response as OD and medium pH change of A. marinus in TRIS-M9 at 25°C at four Ni concentrations.


5.0
4.0
3.0
2.0
1.0
OD (—)
8.0
7.0
6.0
5.0
4.0
pH (---)
50
100
150
200
250
300
350
Time, h
• 0 Ni
▪ 5 X 10-5 M Ni
✧ 5 X 10-4 M Ni
▫ 1 X 10-3 M Ni

growth curve reaching a pH of less than 4. At 5 X 10^{-4}M Ni the OD peak was comparable to that in 5 X 10^{-5}M Ni, as was the growth rate, but growth commenced only after an extended lag phase. Even at as high a Ni concentration as 1 X 10^{-3}M Ni there was growth, though at a slower rate and reduced yield occurring after a yet further extended lag.

Table 17 presents the growth parameters derived from the growth curve data for all ten Ni concentrations used. At a Ni concentration as high as 1 X 10^{-3}M the OD_{MAX} was 25 % of the control and the DT had increased to four times the control. By contrast, in 2216E LN medium with 1.0 ml EQ inoculum at 1 X 10^{-3}M Ni the OD_{MAX} (Fig. 12) was only 14 % of the control and the doubling time (Fig. 11) was increased by a factor of 6 (this Ni concentration was chosen for ready comparison, but note that in that 2216E LN experiment the 1 X 10^{-3}M Ni DT was anomalously low compared to its neighbors, and the real increase in DT might have been 20 fold or more). Even in controls doubling times in TRIS-M9 medium were much slower than in 2216E LN. This indicated perhaps that the organism, although capable of growth on glucose, was not capable of as rapid a metabolic rate when deprived of specific preformed metabolic intermediates. It was noted in the case of 2216E LN medium that the range of Ni concentrations over which the toxic response occurred was 10 to 20 fold. In the case of TRIS-M9 medium the minimum inhibitory dose was considered to be 1 X 10^{-5}M Ni (Table 17), and appreciable growth did occur at the highest Ni concentration tested, 5 X 10^{-3}M, giving a range of 500-fold or greater.

Table 17. Growth parameters of A. marinus in TRIS-M9 medium, 1.0 ml EQ unwashed inoculum.

Ni, M	Doubling time, h	OD_{MAX}	Lag, h
0	7.97	5.2	10
5×10^{-7}	6.15	4.2	9
1×10^{-6}	6.90	6.2	11
5×10^{-6}	7.99	5.3	11
1×10^{-5}	7.34	3.1	11
5×10^{-5}	7.99	2.5	11
1×10^{-4}	21.64	3.4	22
5×10^{-4}	18.27	3.0	88
1×10^{-3}	30.02	1.3	232
5×10^{-3}	26.81	0.22	370

Particularly interesting was the pattern of lag times shown in Table 17. Above a limiting Ni concentration (5×10^{-5}M Ni) the lag time, which was essentially constant from 0 Ni up to that value, began to show a very sharp increase. Fig. 26 is the lag time plotted against the Ni concentration. The plot is highly linear ($r=-0.991$; 99 % significance level). When plotted as log of Ni versus log of lag time (not shown), the slope of the curve (0.983) approached unity, thus there was a 10 fold increase of lag time for each 10 fold increase of Ni concentration. Repetitions of experiments like that above gave very similar patterns of lag time response (Fig. 27). At Ni concentrations greater than 5×10^{-3}M Ni which did not give growth within the time span of the experiment (Table 17), there were still viable A. marinus cells in the medium detectable by streading onto 2216E LN agar.

Consideration of the linear relationship between lag and Ni concentration lead to the hypothesis that upon initial exposure to Ni concentrations greater than the minimum inhibitory concentration the cells were inhibited from division, but remained viable and metabolically active. A mechanism acting at a rate independent of the Ni concentration acted to cause a derepression of division after a time period which was directly proportional to the Ni concentration. Such a phenomenon could arise both by intracellular (cell adaptation or repair of metal-induced lesions) and extracellular (reduction of effective medium Ni concentration, as by precipitation, uptake, or complexation) mechanisms.

Fig. 26. Lag time (h) versus Ni concentration (M) of A. marinus grown in TRIS-M9 medium (1.0 ml EQ inoculum). $r = 0.991$, $m = 1.49 \times 10^{-5}$, $b = -55.4$

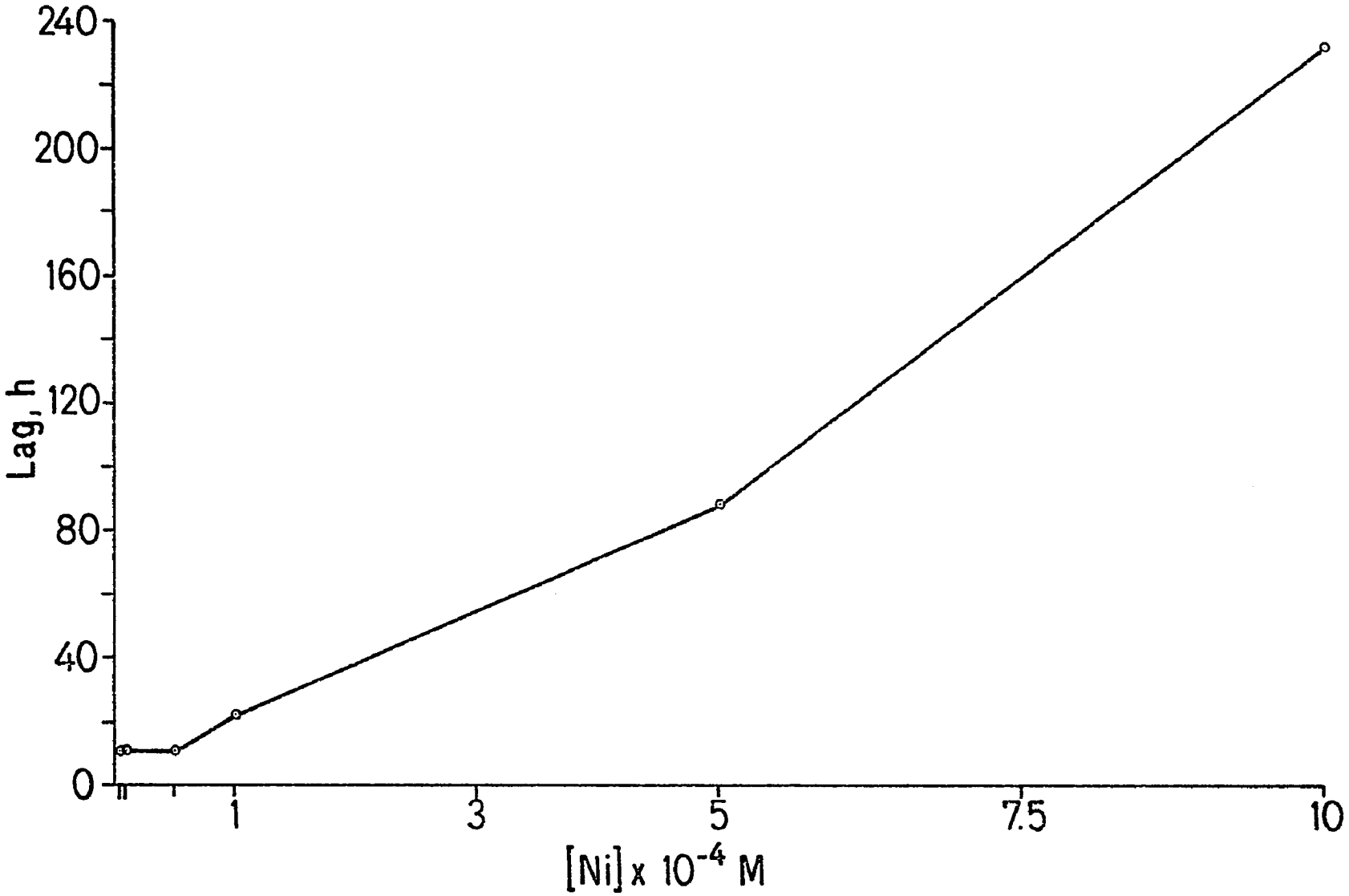


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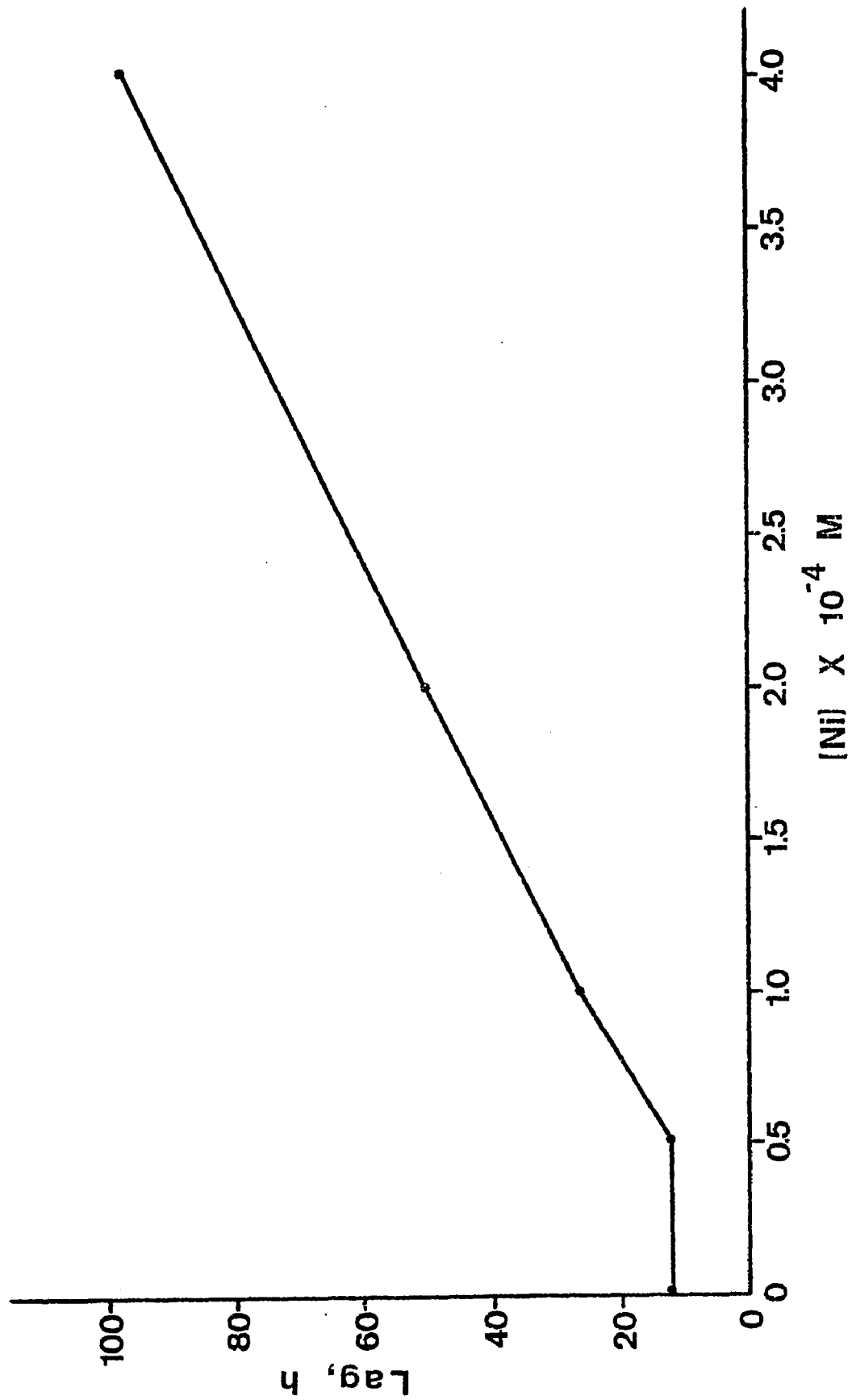
Fig. 27. Lag time (h) versus Ni concentration (M) of A. marinus grown in TRIS-M9 medium (1.0 ml EQ unwashed inoculum). $r = 0.9995$, $m = 2.38 \times 10^{-5}$, $b = 1.39$.


Lag, h
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
[Ni] X 10^-4 M

In addition to the differences in the quantitative growth response of A. marinus to Ni in TRIS-M9 as compared to 2216E LN, there were also differences in the morphological response of the bacteria. Conversion of all of the cells exposed to partially inhibitory concentrations of Ni to greatly enlarged pleomorphic vacuolated cells, as occurred in 2216E LN, was not seen in TRIS-M9 medium. The great majority of cells remained morphologically undifferentiated though there was a tendency for the cytoplasm of the cells to become grainy by phase contrast, particularly at higher Ni concentrations. Cells at those higher Ni concentrations became slightly enlarged (to 1.5 to 2 X 3 to 4 μm).

In initial experiments with TRIS-M9 medium considerable effort was exerted looking for megalomorphic cells which were believed to be an intrinsic part of the A. marinus - Ni interaction. At higher Ni concentrations (5×10^{-4} and 1×10^{-3}M Ni) it was possible to find 2216E LN-type megalomorphic cells by examining the large flocs of detached wall growth in the medium after extended incubation. The megalomorphic cells were embedded in the flocs and arose due to the occurrence of an "A. marinus extract" medium within the cell flocs and were considered as artifacts. Megalomorphy may not be a central feature of the A. marinus - Ni interaction but rather a response limited to cells growing in the presence of preformed metabolic intermediates.

The TRIS-M9 medium experiments were performed using an unwashed inoculum and as considerable difficulty was experienced in reproducing the growth response of A. marinus

in TRIS-M9, the possibility was considered that carry over from the inoculum culture might have effected the growth reproducibility, although that effect had already been shown to be minor in 2216E LN medium. Two flasks each of TRIS-M9 with 0 and 4 X 10^{-4}M Ni were prepared. One flask of each Ni concentration was inoculated with 1.0 ml EQ of an unwashed TRIS-M9 starter culture and the other pair of flasks with 1.0 ml EQ of the same culture after centrifugation at 10,000 rpm and resuspension of the cell pellet in sterile 75 % L&FASW. The growth in the four flasks was monitored and the results presented in Table 18, as OD readings and as calculated growth parameters. There was an effect due to inoculum wash, particularly at 4 X 10^{-4}M Ni in which the OD of the washed culture was lower than the unwashed culture through much of its growth. The difference was minor, however, as seen from the growth parameters which were essentially identical. As with 2216E LN medium, there was a minimal effect on A. marinus growth in TRIS-M9 medium due to inoculum wash.

The growth response of A. marinus in TRIS-M9 was different both from that in 2216E LN and from that which had been expected. Rather than showing Ni toxicity at a lower concentration than in 2216E LN, A. marinus grew in TRIS-M9 at Ni concentrations higher than those giving complete inhibition in 2216E LN. Further, it was difficult or impossible to establish an upper limit of Ni concentration allowing growth in that medium. At the upper Ni concentrations growth was not limited by the medium or the intrinsic response of the organism but rather by time lag. By extrapolating the curve of lag time

Table 18. Growth as OD of A. marinus in TRIS-M9 medium and with 4 X 10^{-4}M Ni, 1.0 ml EQ washed or unwashed inoculum.

		Time, h									
Treatments		0	2	4	7	11	24	48	72	96	120
Unwashed	0	0.000	0.000	0.008	0.002	0.551	0.92	1.96	4.09		
Washed	0	0.009	0.006	0.020	0.026	0.445	0.83	2.14	4.77		
Unwashed	4X10^{-4}M	0.000	0.003	0.006	0.000	0.016	0.092	0.482	0.769	0.92	1.47
Washed	4X10^{-4}M	0.004	0.000	0.009	0.002	0.019	0.045	0.266	0.469	0.548	0.780

		144	192	Doubling time, h	OD_{MAX}	Lag, h
Unwashed	0			16.4	4.09	7
Washed	0			14.4	4.17	7
Unwashed	4X10^{-4}M	3.24	3.50	10.0	3.50	c.20
Washed	4X10^{-4}M	1.53	3.51	10.3	3.51	c.20

versus Ni (Fig. 26), it would be expected that if a culture took 400 h to show growth at 1 X 10^{-3}M Ni, it would require 800 h (one month) to show growth at 2 X 10^{-3}M, 1600 h at 4 X 10^{-3}M, etc. It was impossible to maintain cultures for those extended incubation times. Growth when it occurred, was still to substantial OD. There was no reason to infer that growth would not ultimately occur at Ni concentrations of 5 X 10^{-3} or even 1 X 10^{-2}M though there certainly must be some upper limit at which Ni is irreversibly toxic to A. marinus.

One of the assumptions made concerning Tris buffer was incorrect. It is a moderately strong metal-chelating agent (Cu binding constant 11.1 as compared to EDTA 18.0; Bai and Martell, 1969, cited in Anderson and Morel, 1978). Further, Tris is not a particularly good buffer in the pH range used, as the 7.4 starting pH is near the lower end of its effective buffer range (Tris pK_a= 8.30).

Other buffers claimed to have low or negligible metal-binding constants (Gueffroy, 1975) which had effective buffer ranges for seawater were evaluated. HEPES (pK_a 7.55) and PIPES (pK_a 6.80) gave superior buffering as compared to Tris but in their respective M9 media, A. marinus gave growth responses which were indicative of some metal-binding by the buffers (data not shown). It was indicative of the very strong acid production by A. marinus from glucose that even with HEPES buffer the pH was reduced to 5.1 and with PIPES to 5.9 at the end of growth. Based on the HEPES data, a calculated 0.00134 mole of H^+ ion was produced from the 0.00277 mole of

glucose initially present. Evidently a significant fraction of the substrate was metabolized to an organic acid.

Unbuffered M9

The trial of buffers other than Tris had included evaluation of unbuffered-M9 medium. It gave essentially the same pattern of Ni response as had TRIS-M9 medium but the range of Ni concentrations over which toxicity was expressed shifted downward. The minimum inhibitory concentration of Ni in UB-M9 was 5×10^{-5}M versus 2×10^{-4}M for TRIS-M9 in that experiment. Owing to the apparent medium conditioning during the lag, the lag phase had become the focus of the research and buffers were dispensed with entirely as the pH drop occurred after the initiation of growth and after any medium conditioning or cell adaptation to Ni occurred.

The growth response of A. marinus for a typical experiment is shown in Fig. 28. Note that while Ni concentrations as low as 5×10^{-6}M gave increased lag times relative to the 0 Ni control, the growth rates and maximum OD values for the cultures up to at least 7×10^{-5}M Ni were roughly similar. This is more clearly seen in Table 19 conducted in December, 1976. The doubling time increased by less than half between the 0 Ni and 1×10^{-4}M Ni cultures, and the OD_{MAX} is essentially identical for the nine Ni concentrations which gave growth. By contrast, the lag time increased with each Ni concentration. No values are given for 2×10^{-4}M Ni because those flasks did not start logarithmic growth during the monitoring period (220 h).

Fig. 28. The growth as OD of A. marinus in UB-M9 medium, 1 ml EQ washed inoculum at nine Ni concentrations.

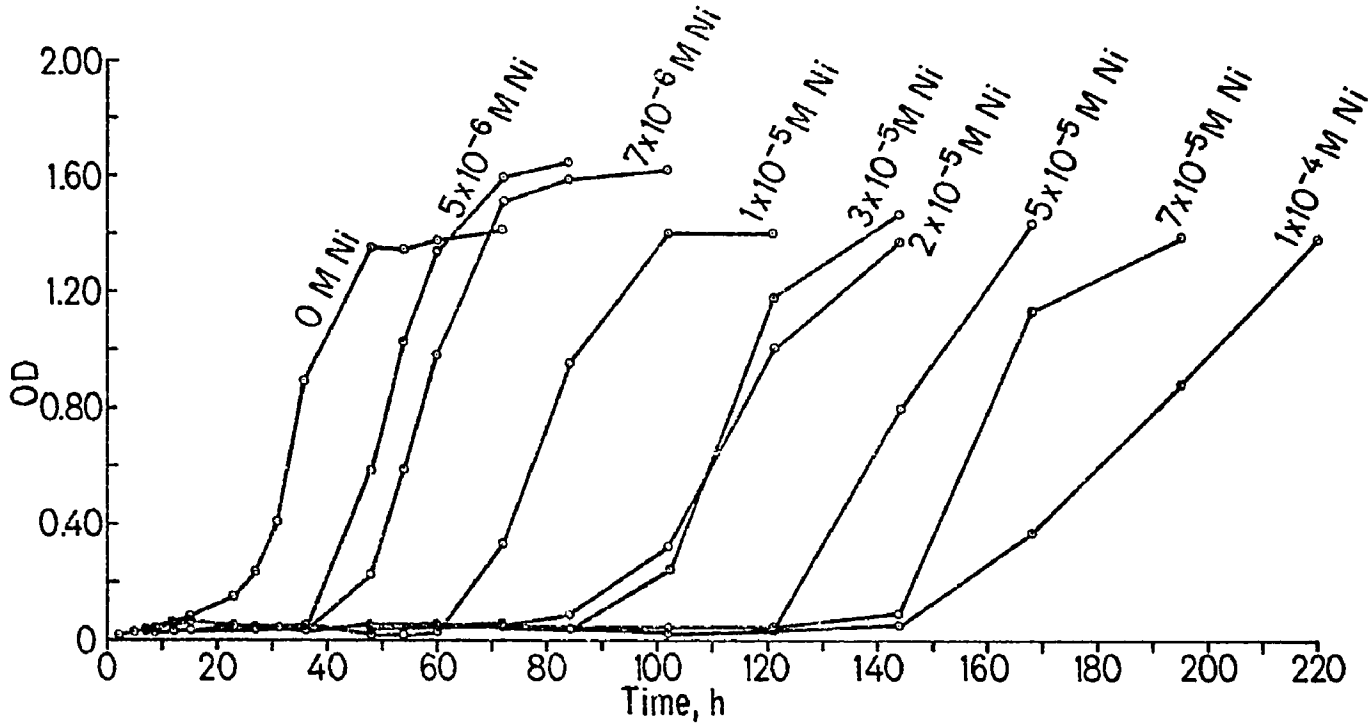

2.00
1.60
1.20
0.80
0.40
0
OD
0
20
40
60
80
100
120
140
160
180
200
220
Time, h
0 M Ni
5x10-6M Ni
7x10-6M Ni
1x10-5M Ni
3x10-5M Ni
2x10-5M Ni
5x10-5M Ni
7x10-5M Ni
1x10-4M Ni

Table 19. Growth parameters of A. marinus in UB-M9 medium at 10 different Ni concentrations (1.0 ml EQ inoculum).

Ni, M	Doubling time, h	OD_{MAX}	Lag, h
0	5.69 ± 1.35[b]	1.41 ± 0.83	11.3 ± 1.1
5×10^{-6}	3.09 ± 0.315	1.64 ± 0.17	38 ± 2.0
7×10^{-6}	4.33 ± 1.09	1.62 ± 0.18	42 ± 5.3
1×10^{-5}	7.91 ± 1.79	1.41 ± 0.14	60 ± 0
2×10^{-5}	9.40 ± 0.218	1.37 ± 0.15	80 ± 6.9
3×10^{-5}	6.28 ± 1.62	1.46 ± 0.036	84 ± 0
5×10^{-5}	4.68 ± 0.897	1.43 ± 0.025	127 ± 11
7×10^{-5}	6.30 ± 2.73	1.39 ± 0.15	143 ± 7.5
1×10^{-4}	7.80 ± 3.09	1.38 ± 0.12	160 ± 21
2×10^{-4}	-	-	DNG[a]

[a]DNG - Did Not Grow.

[b]Mean plus or minus one standard deviation, three replicate flasks.

The lag data for four sets of UB-M9 experiments conducted over a year interval are summarized in Table 20. For each experiment the linear regression of lag time versus Ni concentration was calculated. For each set of data the regression was a linear function at or above the 99 % confidence level as was the case with the lag time - Ni relationship with TRIS-M9 medium. However in the case of UB-M9 medium, the slope of the log - log plot was less than 1 (0.486 to 0.765) with a mean of 0.58. In these experiments a ten fold increase of Ni concentration caused a 3.8-fold increase of lag time rather than the ten-fold increase as with TRIS-M9 medium.

There was considerable variation between the four experiments in Table 20. Thus, the minimum inhibitory dose varied from 2 X 10^{-6}M Ni (May 1977) to 2 X 10^{-5}M Ni (July 1976). This type of variation of the growth response occurred at sporadic intervals during the study. The differences between the four experiments were not the result of random variations between the individual flasks of an experimental series, but rather were the result of a systematic variation. That is clear from examination of the data of Table 19. In that experiment with triple replication of the experimental flasks, the coefficient of variation of the lag time at each Ni concentration was 13 % or less, far less than the inter-set variation. The variations between experiments were not of a random nature either. Rather, after having established the range of Ni - A. marinus response, for a period of time the response pattern was reasonably predictable. Then abruptly, experiments based on an expected lag time failed and it was necessary to

Table 20. Lag time (h) versus Ni concentration (M) for *A. marinus* grown in UB-M9 medium (1.0 ml EQ inoculum), four separate experiments.

Ni, M	July 1976[a]	Sept. 1976[a]	Dec. 1976[b]	May, 1977[b]
0	7	10	11	20
1×10^{-6}				20
2×10^{-6}				30
5×10^{-6}	8		38	30
7×10^{-6}			42	50
1×10^{-5}	8	13	60	
2×10^{-5}	12		80	120
3×10^{-5}			84	
4×10^{-5}	20			
5×10^{-5}		54	127	150
7×10^{-5}			143	
8×10^{-5}	24			
1×10^{-4}	24	74	160	290
1.5×10^{-4}	30	130		
2×10^{-4}	35	130		
3×10^{-4}	70	190		
4×10^{-4}	96			

[a]unwashed inoculum; [b]washed inoculum

Regression analysis of log lag time versus log [Ni].

r	correlation coefficient	-0.860	-0.995	-0.990	-0.957
m	slope	-0.516	-0.765	-0.486	-0.538
b	y-intercept	3.59	4.99	4.16	4.48
df	degrees of freedom	7	7	6	4
Confidence level		99%	99.9%	99.9%	99%

reestablish the range of Ni concentrations over which the toxicity response was occurring. In later experiments, it was accepted as a standard procedure that for each batch of UB-M9 medium a set of flasks was prepared with a range of Ni concentrations which was inoculated and its growth monitored prior to inoculating the main experiments to determine the response range for that medium batch.

A number of variables were examined to determine if they might have been contributing to the variability observed. Of the four experiments in Table 20, two were with unwashed inocula (July 1976 and Sept 1976), and two were with washed inocula (Dec 1976 and May 1977), perhaps contributing to their differences. Two growth sets were prepared from a single batch of UB-M9 medium with Ni concentrations from 0 to 4 X 10^{-4}M. One set was inoculated with 1.0 ml EQ of an unwashed A. marinus starter and the other with an equal amount of the same starter culture after washing. From the recorded growth curve of each flask, the growth parameters were calculated (Table 21). The growth curves for the two inocula roughly paralleled each other, particularly at the lower Ni concentrations and up to 2 X 10^{-5}M Ni there were essentially no differences in the growth parameters attributable to washing (the very slow growth rate at 1 and 2 X 10^{-5}M Ni, washed, were anomalies due to the spacing of sampling times). At higher Ni concentrations the washed inoculum tended to give somewhat longer lag times. As these differences were of greater magnitude than the differences among flasks of replicates (see Table 19), there were considered to have been real differences due to inoculum wash. The

Table 21. Effect of washed or unwashed inoculum on growth of A. marinus in UB-M9 medium.

Inoculum	Ni, M	Doubling time, h	OD_{MAX}	Lag, h
Washed	0	4.23	1.31	15
	1×10^{-6}	3.29	1.27	15
	5×10^{-6}	3.21	1.45	20
	1×10^{-5}	10.9	1.02	30
	2×10^{-5}	15.8	1.44	50
	5×10^{-5}	8.78	1.55	80
	1×10^{-4}	8.16	1.46	125
	2×10^{-4}	-	DNG[a]	-
	4×10^{-4}	-	DNG	-
Unwashed	0	4.90	1.20	15
	1×10^{-6}	5.28	1.18	15
	5×10^{-6}	3.05	1.25	20
	1×10^{-5}	7.47	1.19	27
	2×10^{-5}	9.83	1.27	50
	5×10^{-5}	7.90	1.47	60
	1×10^{-4}	13.4	1.70	80
	2×10^{-4}	8.02	0.781	120
	4×10^{-4}[b]	-	-	260

[a]DNG - Did Not Grow

[b]Last point was taken just as cells went into log phase.

Regression of -log Ni vs log lag time

washed		unwashed	
r=-0.984	df=4	r=-0.979	df=6
m=-0.506	b=4.07	m=-0.478	b=3.89
$P \leq 0.001$		$P \leq 0.001$	

regression analysis of the lag data (Table 21) indicated that a very similar lag time - Ni response occurred in the two treatments giving a statistically significant linear relationship between Ni concentration and lag time. While the washing of the inoculum gave a significant effect in UB-M9, the effect, as was the case with 2216E LN and TRIS-M9, was minor, and was insufficient to account for the differences between different UB-M9 experiments. In subsequent experiments washed inocula were used.

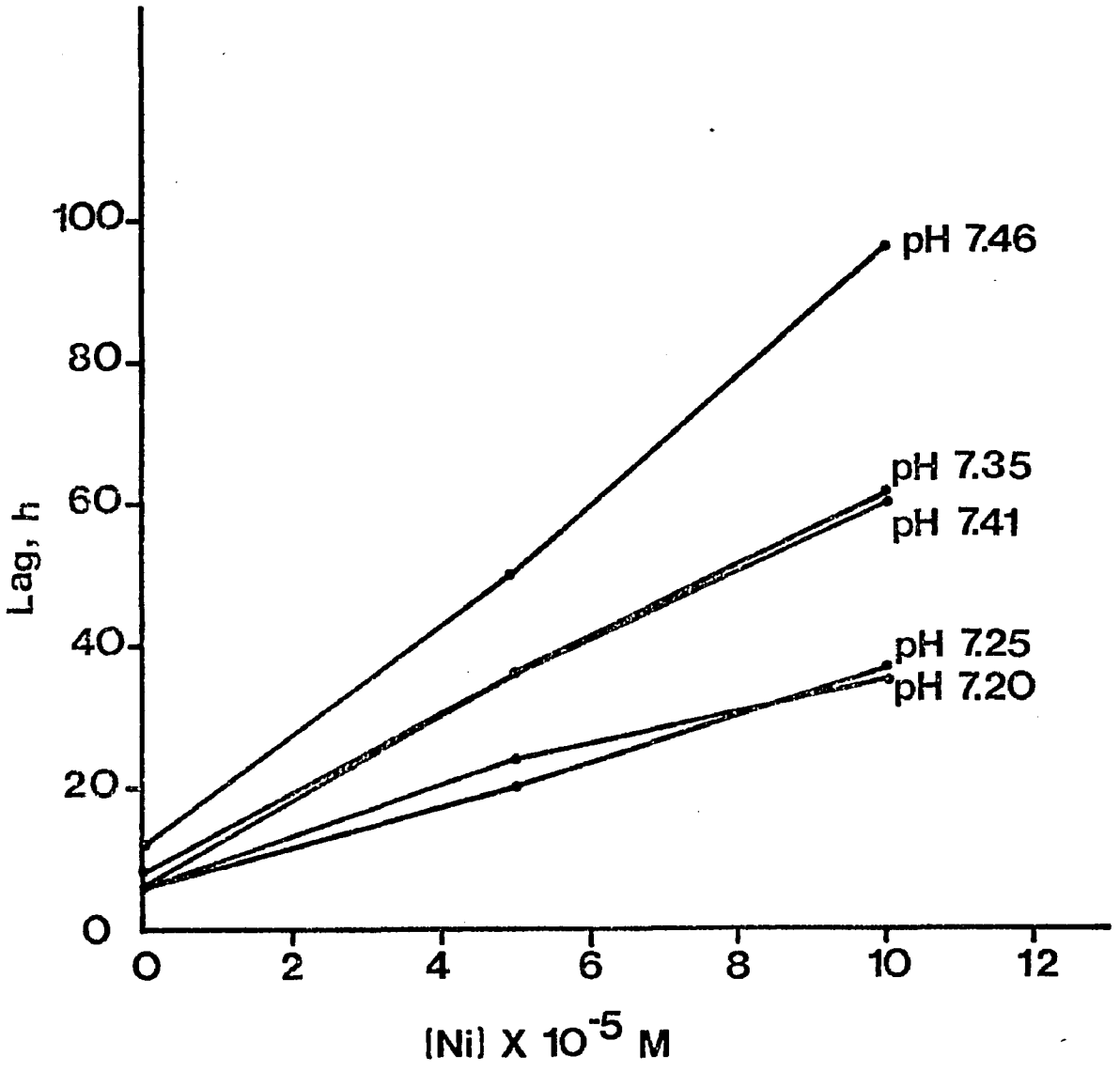
During the procedure of preparation of UB-M9 the pH adjustment was designed for a final pH of 7.4, but inherent variation and the extreme sensitivity of the unbuffered medium to small amounts of acid led to variation in the starting pH of each batch of medium (pH 7.2 to 7.5), though the procedure of adding the sterile 0.05 N HCl via a repeating dispensor kept the variation among flasks of a given medium batch to c. 0.05 pH unit. To examine if the variation of starting pH contributed to the variation between batches of UB-M9, an experiment was set up in which five identical sets of UB-M9 medium were prepared with added Ni concentrations of 0, 5 X 10^{-5}, and 1 X 10^{-4}M. From the results of titrations of samples of the medium, acid additions were made to bring one of the sets of flasks to each of the following final pH values: 7.20, 7.25, 7.35, 7.40, 7.46. The flasks were inoculated with 1.0 ml EQ of a washed inoculum, and the growth monitored (Table 22).

The doubling time and OD_{MAX} did not show apparent systematic variations with starting pH. The lag time, however, did show a consistent pattern of change with pH (Fig. 29) in

Table 22. Effect of initial medium pH values on the growth of A. marinus in UB-M9 medium at three Ni concentrations.

Initial pH	Ni, M	Doubling time, h	OD_{MAX}	Lag, h
7.20	0	3.66	1.30	6
	5×10^{-5}	17.8	0.90	24
	1×10^{-4}	15.8	0.87	35
7.25	0	3.71	1.28	6
	5×10^{-5}	20.3	0.97	20
	1×10^{-4}	20.9	0.92	36
7.35	0	3.39	1.16	6
	5×10^{-5}	12.0	1.32	36
	1×10^{-4}	13.1	1.04	60
7.41	0	3.72	1.08	8
	5×10^{-5}	12.8	1.10	36
	1×10^{-4}	26.7	0.86	60
7.46	0	3.24	1.18	12
	5×10^{-5}	15.1	0.91	50
	1×10^{-4}	6.84	0.76	96

Fig. 29. Lag time (h) versus Ni concentration (X 10^{-5}M) for A. marinus grown in UB-M9 medium of five initial pH values.


100
80
60
40
20
0
Lag, h
0
2
4
6
8
10
12
[Ni] X 10^{-5} M
pH 7.46
pH 7.35
pH 7.41
pH 7.25
pH 7.20

which the slope of the lag versus Ni plot decreased with decreasing pH. Thus at an initial pH of 7.20 the slope of the log - log plot was -0.46, while at an initial pH of 7.46 the slope was -0.94. The degree of variation due to pH did not approximate the magnitude of variation between different batches of medium. Also, the starting pH of the flasks in the four growth series cited above were: July 1976, 7.20; Sept 1976, 7.41; Dec 1976, 7.50; May 1977, 7.41. The variation of pH did not correlate with the changes in the Ni toxicity range between experiments. Further, variation of medium batch pH was a random factor whereas the shifts of Ni toxicity range, while unpredictable, occurred systematically. Thus medium batch initial pH had an appreciable effect on the growth response of A. marinus in UB-M9 but the observed magnitude of the effect was insufficient to account for the variation observed between UB-M9 growth curve sets.

Several other potential sources of variation were also investigated. Variations of the source of nutrient constituents of UB-M9 medium gave statistically insignificant differences of the DT, OD_{MAX}, and lag times for both 0 Ni and 5×10^{-5}M Ni concentrations. Another source of variation which was considered was the occurrence of contamination or mutation of the A. marinus LAB stock culture. The use of a freshly reactivated culture from the frozen storage cultures never gave a significantly different response from the stock culture previously in use.

None of the factors effecting culture response variability which had been studied were capable of solely having

caused the shifts of A. marinus - Ni toxicity range observed; nor did several factors acting in concert cause the variation, owing to the non-random variations of the observed medium response. It is more probable that the variations were the result of the interaction of the very sensitive A. marinus - UB-M9 system with variations of glassware wash, artificial seawater composition, or other unidentified variables. However, as was emphasized above, this system, though delicately balanced was useable experimentally if the precaution was taken that the Ni response range for each medium batch was determined prior to its use for experiments, and if experiments were designed to be internally controlled, with comparisons between experiments performed with different medium batches being made with great caution.

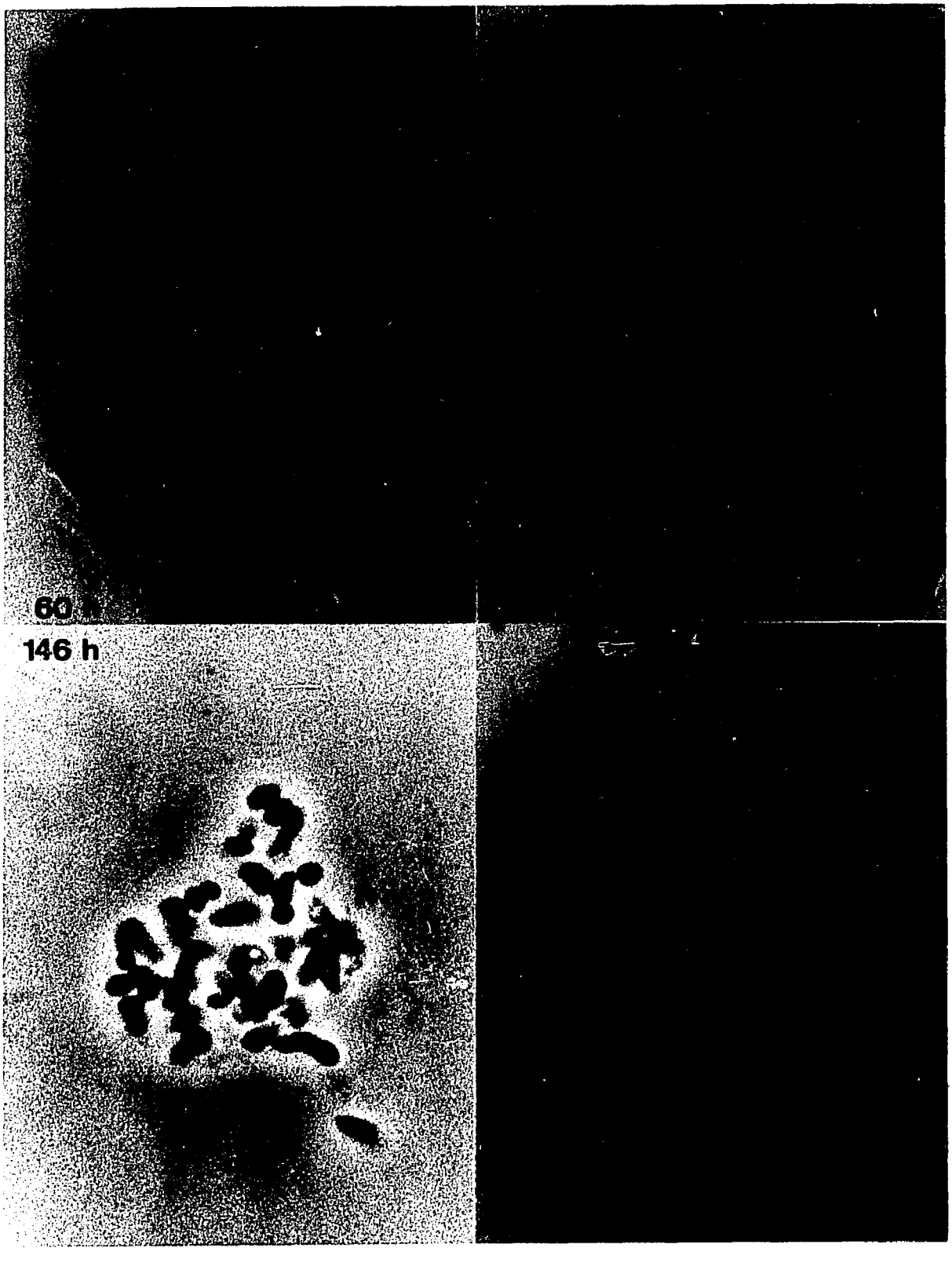
It had previously been noted that the megalomorphic response of A. marinus was lacking in TRIS-M9 medium. The growth of A. marinus in UB-M9 medium is shown in Fig. 30. The occurrence of cell clumping, which was a source of imprecision in the measurement of culture biomass in 2216E LN medium, was prominent in UB-M9 and buffered M9 media. Though not well shown by these micrographs, the cells had a tendency to have intracellular phase-bright granules. When grown in the presence of inhibitory concentrations of Ni (Fig. 31; 1 X 10^{-4}M Ni), the cells, whether in lag (60 and 106 h), log (146 h), or stationary phases (288 h), had essentially identical morphologies, slightly enlarged relative to the 0 Ni control, and notably phase-grainy (Fig. 31c and d). As in TRIS-M9 medium, the megalomorphic response to Ni was totally lacking. In

Fig. 30. A. marinus grown in UB-M9 medium, at 25°C after various times.


10 µm

Fig. 31. A. marinus grown in UB-M9 medium with 1 X 10^{-4}M Ni, at 25°C after various times.

M Ni,



experiments not shown, the same was true of HEPES- and PIPES-M9 media as well.

It was concluded that the pattern of growth of A. marinus with respect to Ni in UB-M9 medium was essentially identical to that in TRIS-M9 medium, with the exception that the range of Ni concentrations over which toxicity was expressed was lower (by about ten fold) in the unbuffered medium, and the growth yield of the bacterium was lower (by a factor of 2 to 3) due to early shutoff of growth due to reduction of the medium pH. The generalized pattern of growth in defined M9-type media was distinctly different from that in the complex 2216E-type media. The cell morphology response was in sharp contrast due to the absence of the megalomorphic response of A. marinus to Ni in the glucose media. The pattern of growth response was also distinctly different. Rather than the 2216E LN-type response in which over a relatively narrow range of toxicant concentrations the growth rate and yield decreased from control levels to 0, with the M9 media over a much wider range of Ni concentrations the growth yield and rate were relatively constant and toxicity was expressed by a lag time which increased proportionately with Ni concentration above a minimum inhibitory dose. Lag times in 2216E LN medium, by contrast, were shorter than in M9 media even for the 0 Ni control and showed little increase even at the most strongly inhibitory Ni concentrations.

As was discussed above with respect to TRIS-M9 medium, the lag phase phenomenon in UB-M9 medium was also interpreted as indicative of either a cell adaptive response to A. marinus

during the lag time, or of the conditioning of the medium by the cells, such that the effective toxicity of the metal was reduced.

A. marinus - Ni Lag Phase Effect

Medium Conditioning Bioassay

To differentiate between the two possible modes of termination of the Ni-induced A. marinus lag phase, several experiments examined further the growth of the organism of which the first was a conditioning bioassay procedure. This was performed by reinoculating a previously inoculated and filter resterilized culture. If the second inoculum showed a reduced lag time, and if the reduction of lag time was proportional to the incubation time of the original inoculum, conditioning of the medium by the first inoculum would be indicated.

Flasks of UB-M9 medium with 0 or 2 X 10^{-5}M Ni added were prepared and inoculated with 1.0 ml EQ of a washed inoculum. At each Ni concentration one of the flasks was monitored by OD throughout its growth which is presented as the control flasks (A and B; Table 23, Fig. 32). After 7 h (0 Ni) or 15 h (2 X 10^{-5}M Ni) the other flasks were removed from the shaker, filter sterilized through 0.4-μm Nuclepore membranes (to remove the primary inoculum), and aseptically transferred to sterile 250 ml flasks. For 0 Ni harvesting was at 70 % of the control lag time, for 2 X 10^{-5}M, it was at 30 % of control lag time. The filter harvested medium (designated 0 or 2 X 10^{-5} PG) was then inoculated with 1.0 ml EQ of a fresh inoculum, and their

Table 23. Medium conditioning bioassay (I).

[Ni], M	Doubling time, h	OD_{MAX}	Lag, h
Control[a]	3.19	1.58	10
Control[a] 2×10^{-5}	11.0	1.55	50
PG[b] 0	8.4	1.24	c.5
PG 2×10^{-5}	6.48	1.08	c.7
PG 1:1 1×10^{-5}[c]	4.60	1.57	c.7
PG 1:1 2×10^{-5}[d]	4.72	1.17	18

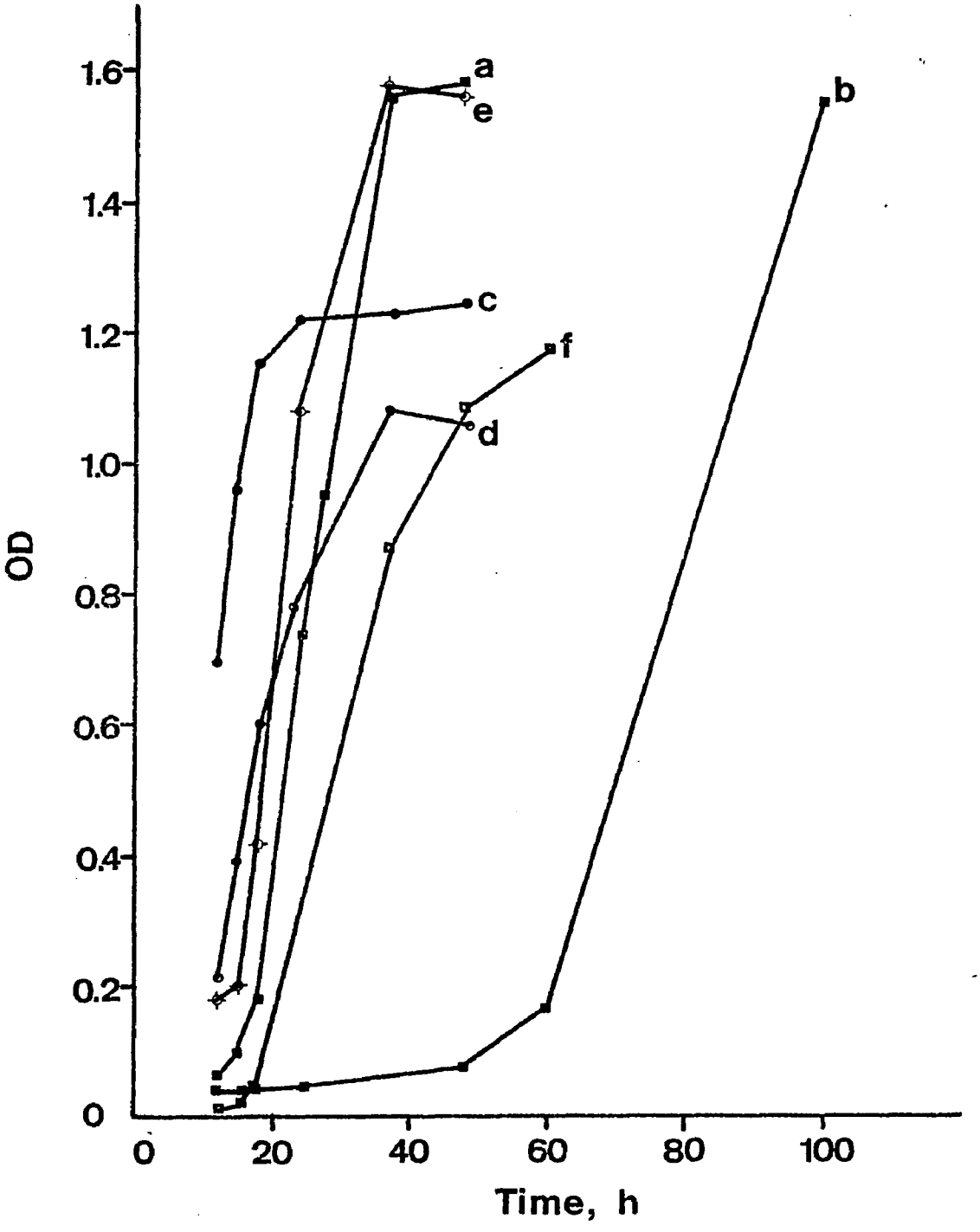
[a]initial growth response

[b]pregrown, harvested by filtration after 7 h (0) or 15 h (2×10^{-5}M) incubation and reinoculated

[c]50 ml 2×10^{-5}M PG + 50 ml UB-M9 medium

[d]50 ml 2×10^{-5}M PG + 50 ml 2×10^{-5}M Ni UB-M9 medium

Fig. 32. Effect of media preconditioning on the growth of A. marinus in UB-M9 medium: a (0 Ni) and b (2 X 10^{-5}M Ni) control flasks showing original growth response; c (0 Ni) and d (2 X 10^{-5}M Ni) preconditioned flasks harvested at 7 and 15 h and reinoculated with fresh 1 ml EQ culture; e (2 X 10^{-5}M Ni) 15 h harvested flask diluted 1:1 with fresh 0 Ni UB-M9 medium and reinoculated; f (2 X 10^{-5}M Ni) 15 h harvested flask diluted 1:1 with fresh 2 X 10^{-5}M UB-M9 medium and reinoculated.


OD
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time, h
a
b
c
d
e
f

subsequent growth monitored (Table 23; Fig. 32). Two other treatments (PG 1:1 1 X 10^{-5} and PG 1:1 2 X 10^{-5}) were prepared by mixing 50 ml of 2 X 10^{-5}M PG broth with either 50 ml of fresh UB-M9 (the former) or 50 ml of fresh 2 X 10^{-5}M Ni UB-M9 broth (the latter). These were also inoculated and their growth monitored (Table 23; Fig. 32). There was a distinct reduction of the lag time of the 0 and 2 X 10^{-5}M Ni PG media compared to their respective controls. The readdition of fresh Ni to the 2 X 10^{-5}M PG broth (PG 1:1 2 X 10^{-5}) reestablished a lag phase of 18 h. The growth curves (Fig. 32) for all treatments were similar, only the length of the lag phases were changed by conditioning of the medium by A. marinus pregrowth. This was indicative of production by the cells of a substance which neutralized the Ni toxicity.

This result was confirmed in a second experiment and extended to include time dependency of the conditioning (Table 24). The basic procedure was as above, with the flasks being used in duplicate to increase precision. In addition, sampling was carried out at intervals during the lag phase of the initial inoculum in order to determine if the conditioning of the medium by the A. marinus cells increased as the length of incubation of the primary inoculum increased.

The initial growth of the organism with 0, 1 X 10^{-5} and 2 X 10^{-5}M Ni (Table 24) showed the expected lag time response. The growth upon reinoculation of the pregrown flasks also conformed to expectations. There was a constant depression of the magnitude of the lag phase of the secondary inoculum during conditioning such that after 48 h (just prior

Table 24. Medium conditioning bioassay (II).

[Ni], M	Doubling time, h	OD_{MAX}	Lag, h
0 Initial[a]	2.29 ± 0.348	1.87 ± 0.06	9.33 ± 1.7
1 X 10^{-5} Initial	3.92 ± 0.683	1.64 ± 0.15	23.7 ± 2.3
2 X 10^{-5} Initial	7.01 ± 1.11	1.41 ± 0.08	51.7 ± 2.9
T_o PG[b]	17.9 ± 12.7	1.07 ± 0.05	26.5 ± 9.2
T_{12} PG	17.6 ± 4.92	0.849 ± 0.298	20.0 ± 0.0
T_{24} PG	13.5 ± 2.05	0.735 ± 0.021	12.0 ± 0.0
T_{36} PG	15.5 ± 2.46	0.683 ± 0.074	10.0 ± 0.0
T_{48} PG	23.1 ± 0.283	0.780 ± 0.085	8 ± 0.0

[a]initial growth response

[b]pregrown, harvested by filtration at time (h) indicated and reinoculated

to growth initiation of the primary inoculum) the lag time of the secondary inoculum (8 h) had fallen to a value equivalent to the 0 Ni control lag time (9.33 h). There was a significant decrease of the secondary lag time as early as c. ½ h after primary inoculation (the approximate time between inoculation and harvesting of the T_o PG flasks).

In this experiment the rate of medium conditioning was not constant during the primary lag phase, perhaps due to release of an endogenous reserve of medium conditioning agent by the inoculum. Unlike the first bioassay experiment, the OD_{MAX} was depressed and the DT somewhat increased in the PG flasks relative to the controls (Table 24). That depression of growth was perhaps indicative of partial exhaustion of the medium nutrients by the primary inoculum.

These two experiments thus showed evidence for the time dependent reduction of Ni toxicity (expressed as reduced lag time of a secondary inoculum). This medium conditioning could have been due to several mechanisms, including precipitation of the Ni as an insoluble salt, the uptake of Ni by the cells of the primary inoculum, or the production of metal-binding organic compounds.

Changes of the concentrations of the major nutrients of UB-M9 medium, perhaps due to uptake by the cells of the primary inoculum, were examined. From the same set of flasks as the experiment immediately above, flasks of 2×10^{-5}M Ni UB-M9 medium harvested at 0, 12, 24, 36, and 48 h incubation of the primary inoculum were analyzed for glucose, phosphate, and ammonium content. The growth of the original culture was

such that shortly after 48 h active growth started. The analysis results, as the mean concentration and concentration as percent of the time 0, ± one standard deviation, are given in Table 25. There were relatively minor changes in glucose (it fell) and ammonium (it rose) during lag. There did appear to be a significant loss of phosphate, although there was a large standard deviation for that determination, which may have given rise to the apparent changes. It had previously (unreported data) been found that reduction of the UB-M9 phosphate concentration to 0.10 g/liter reduced the culture growth yield by c. 50 %. The depressed growth yield in the second set of bioassays (Table 24) may have been due to phosphate uptake by the first inoculum.

The potential existed that the conditioning of the medium which was observed in these experiments was due to the lysis of cells during the process of removing the primary inoculum by membrane filtration. The cell cytoplasm released by such lysis would be a source of a variety of metal-binding compounds. Cells of A. marinus which had been uniformly labeled by growth on 0.2 μCi/ml ^{14}C-glucose (total volume 25 ml) for several generations were washed and used as the inoculum for several generations of subsequent growth on unlabeled glucose and again were washed. The result was a suspension of cells which had all portions of the cell labeled but had minimal amounts of label present as metabolite pools which might be excreted under stress. Aliquots of those cells were filtered through 0.4-μm Millipore filters at varying differential pressures, and the filter and filtrate activity counted

Table 25. UB-M9 medium nutrient concentrations during the lag time of A. marinus.

Time, h	glucose		phosphate		ammonium	
	g/liter	% T_o	g/liter	% T_o	g/liter	% T_o
0	5.2 ± 0.13	100 ± 2.6	0.165 ± 0.0014	100 ± 0.87	0.866 ± 0.016	100 ± 1.81
12	4.8 ± 0.03	92.3 ± 1.3	0.090 ± 0.0077	54.5 ± 4.7	0.913 ± 0.047	111 ± 5.43
24	4.5 ± 0.15	86.5 ± 2.6	0.095 ± 0.029	57.6 ± 17.6	0.881 ± 0.174	102 ± 20.1
36	4.4 ± 0.40	85.5 ± 7.8	0.150 ± 0.055	90.9 ± 33.6	0.970 ± 0.036	112 ± 4.22
48	4.5 ± 0.15	86.5 ± 2.8	0.130 ± 0.041	78.9 ± 24.9	1.00 ± 0.052	115 ± 0.60

by LSC. Filtration differential pressures were varied from 25.4 to 76.2 cm Hg. The remaining cell suspension was then clarified by centrifugation (12,000 X g; 10 min) and the supernatant activity determined. The results are shown in Table 26. The filtrate activity was essentially constant at 1.3 to 1.8 % of the input activity. By contrast the centrifuge supernatant contained 3.7 % of the input activity. Thus, there was essentially no cell leakage during membrane filtration and medium conditioning was not an artifact of filtration.

Effect of Inoculum Size

The degree of dependence of the lag time induced by Ni is an indication of whether the effect is due to cell adaptation or to medium conditioning. If the lag was due to cell adaptation and/or repair of metal induced metabolic lesions, there should be relatively little change in the lag time over a wide range of inoculum sizes (Mitra et al., 1975). By contrast, if the phenomenon was due to conditioning of the medium by the action of metabolically active but division-inhibited cells, a larger inoculum should give a proportionately larger population of cells producing the conditioning agent, and the lag time would be reduced proportionately to the size of the primary inoculum. Similarly, a reduced inoculum would give a proportionately extended lag time.

Experiments to test the effect of variations of inoculum size were performed by preparing multiple sets of UB-M9 broth flasks with a range of Ni concentrations as a single batch. These were simultaneously inoculated with varying A. marinus

Table 26. Quantitation of *A. marinus* cell leakage during vacuum filtration.

Vacuum pressure		Filtrate[b]		Cells[b]	
in Hg	cm Hg	CPM/ml	%[c] Cell Suspension	CPM/ml	%[c] Suspension
10	25.4	15.5	1.55	1088	109
15	38.1	13.0	1.30	1070	107
20	50.8	18.5	1.85	1011	101
25	63.5	14.0	1.40	1520[a]	150[a]
30	76.2	14.0	1.40	1144	114
Cell suspension[d]		37.0	3.70		

[a]anomalously high value due to cell clump.

[b]14 C-labeled cells filtered at indicated differential pressure

[c]percent of initial activity per ml

[d]remaining suspension centrifuged (10 min 12,000 μg)

inoculum sizes from a single starter, and the growth monitored. The results of such an experiment with Ni concentrations of 0, 5×10^{-6}, 1×10^{-5}, and 2×10^{-5}M Ni and inoculum sizes of 0.1 to 5.0 ml EQ (washed) are shown in Table 27 as growth parameters. With the exception of an anomalously low value for 5.0 ml inoculum, 2×10^{-5}M Ni, there was no systematic variation of the OD_{MAX} due to inoculum size. Similarly, there was no systematic variation in the growth rate with inoculum size. There was by contrast a definite effect of inoculum size on the lag time of the culture. A linear relationship between Ni concentration and lag time occurred with each inoculum size. Fig. 33 plots the inoculum size against the lag for the 4 Ni concentrations used. The magnitude of the inoculum size effect increased with increasing Ni concentration. However, even at 2×10^{-5}M Ni the lag time varied by a factor of only 4 over the 50:1 range of inoculum sizes, considerably less than expected from such an inoculum range if lag and inoculum were inversely proportional.

To further study the inoculum size effect at higher medium Ni concentrations, an experiment like that above was set up using Ni concentrations of 0, 1, 2, 5, and 10×10^{-5}M and inoculum sizes of 0.2, 1.0, and 2.0 ml EQ washed. Table 28 presents the growth parameters for this experiment. Again with the exception of two anomalous values (0.2 ml, 1×10^{-4}M, OD_{MAX}; 1.0 ml, 1×10^{-4}M, DT), the DT and OD_{MAX} showed no persistent variation with inoculum size. There was a definite effect on the lag time due to inoculum size. The slope of the Ni vs lag curve (Table 28) increased as the inoculum size

Table 27. Effect of inoculum size on the growth of A. marinus in UB-M9 medium with different Ni concentrations (I).

Inoculum size, ml EQ	[Ni], M	Doubling time, h	OD_{MAX}	Lag, h
0.1	0	7.48	1.8	30
	5×10^{-6}	7.19	1.24	36
	1×10^{-5}	7.03	1.58	36
	2×10^{-5}	8.72	1.31	100
0.2	0	7.73	1.78	20
	5×10^{-6}	3.04	1.39	30
	1×10^{-5}	13.4	1.42	36
	2×10^{-5}	8.97	1.34	95
0.5	0	8.15	1.43	12
	5×10^{-6}	5.02	1.25	50
	1×10^{-5}	12.9	1.40	55
	2×10^{-5}	7.70	1.39	95
1.0	0	7.69	1.73	10
	5×10^{-6}	5.45	1.33	30
	1×10^{-5}	8.38	1.45	40
	2×10^{-5}	6.59	1.33	75
2.0	0	5.29	1.47	25
	5×10^{-6}	4.94	1.21	30
	1×10^{-5}	6.02	1.46	50
	2×10^{-5}	11.8	1.20	75
5.0	0	6.09	1.33	8
	5×10^{-6}	4.50	1.32	12
	1×10^{-5}	7.58	1.21	25
	2×10^{-5}	15.2	0.663	25

Fig. 33. Lag time (h) versus inoculum size for different Ni concentrations of A. marinus grown in UB-M9 medium, 25°C.

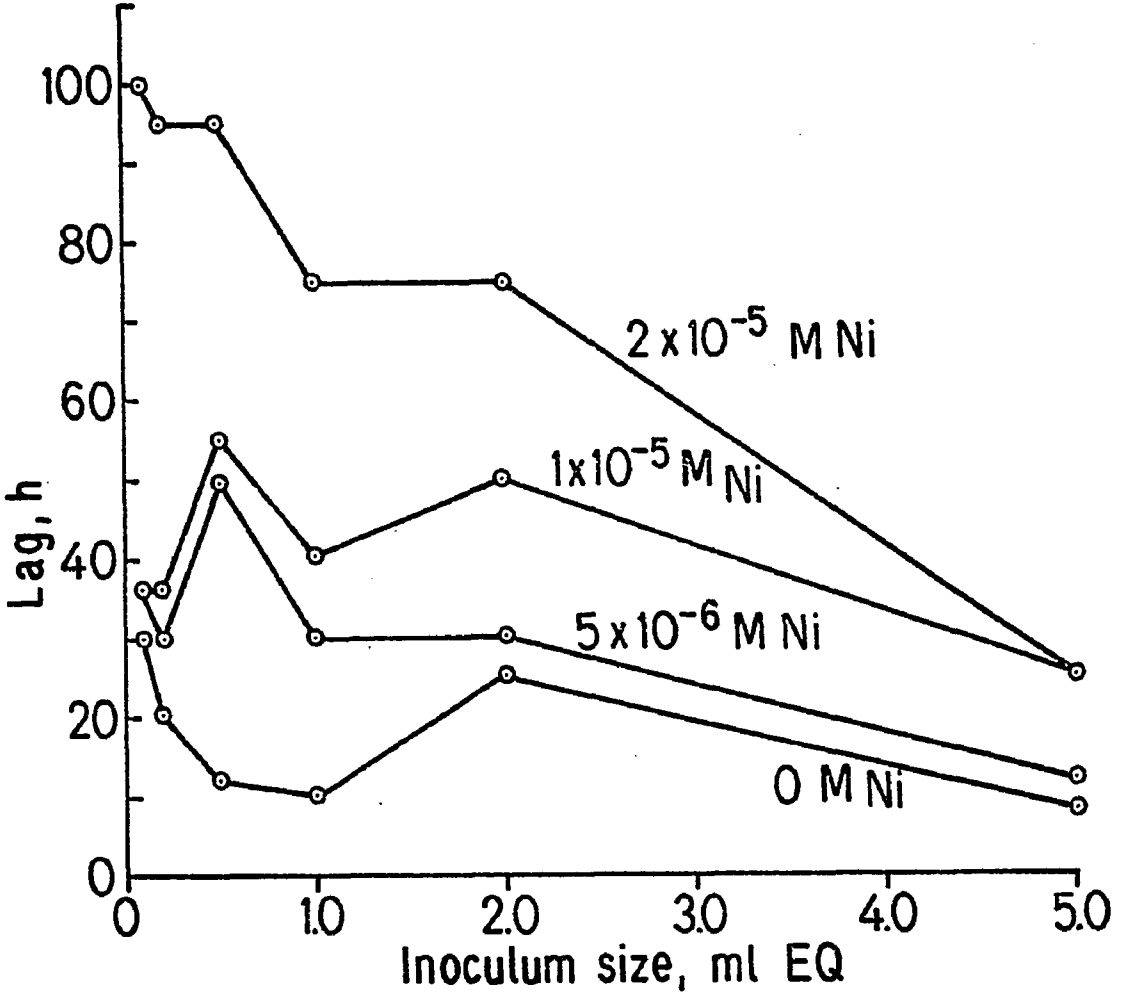

100
80
60
40
20
0
Lag, h
2 x 10⁻⁵ M Ni
1x10⁻⁵ M Ni
5 x 10⁻⁶ M Ni
0 M Ni
0
1.0
2.0
3.0
4.0
5.0
Inoculum size, ml EQ

Table 28. Effect of inoculum size on the growth of A. marinus in UB-M9 medium with different Ni concentrations (II).

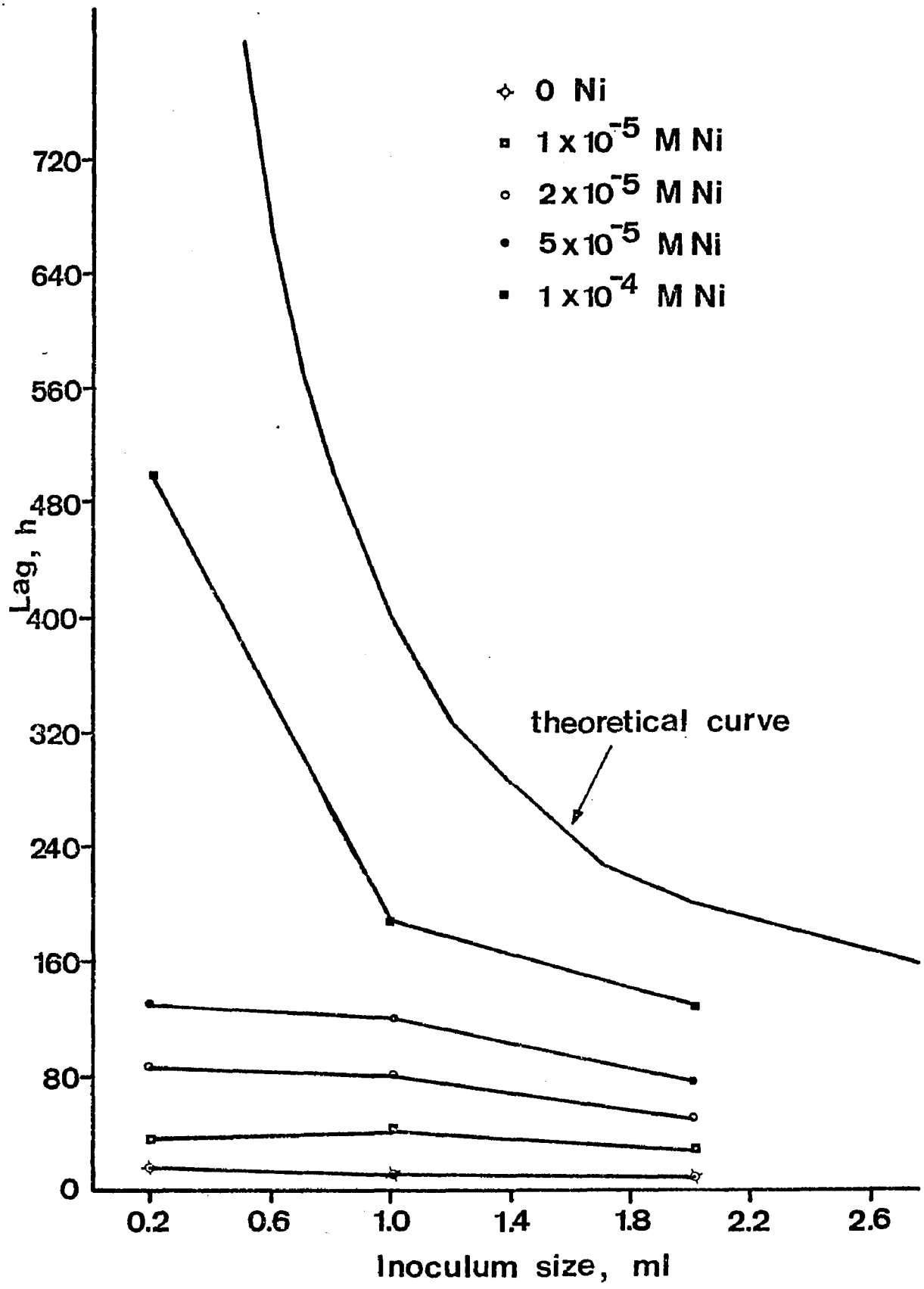
Inoculum, ml EQ	[Ni], M	Doubling time, h	OD_{MAX}	Lag, h
0.2	0	4.08	1.58	17
	1×10^{-5}	2.99	1.67	36 ± 8
	2×10^{-5}	7.42	1.25	85 ± 18
	5×10^{-5}	5.72	1.7	130 ± 19
	1×10^{-4}	8.20	0.616	500 ± 140
1.0	0	5.98	1.45	12
	1×10^{-5}	6.99	1.27	42
	2×10^{-5}	8.13	1.08	80 ± 14
	5×10^{-5}	7.59	0.95	121 ± 32
	1×10^{-4}	14.8	1.85	192 ± 68
2.0	0	4.17	1.36	10
	1×10^{-5}	7.43	1.15	30
	2×10^{-5}	8.24	1.00	55 ± 7
	5×10^{-5}	7.27	1.75	75
	1×10^{-4}	5.28	1.46	130 ± 19

decreased. Fig. 34 plots the inoculum size versus the lag time for the Ni concentration. A theoretical curve based on a doubling of lag time for each halving of inoculum was added. At the highest Ni concentration the experimental curve approached the theoretical response.

Thus there was a definite dependence of the culture lag time on the inoculum size of A. marinus which argued for medium conditioning rather than cell adaptation. At the Ni concentrations studied there was a smaller effect due to inoculum size than would be predicted on theoretical grounds. The magnitude of the inoculum size effect increased as the Ni concentration of the medium increased approaching the theoretical response at the highest Ni concentration (1×10^{-4}M Ni) studied.

The observation was made that agar slants of Ni-containing UB-M9 medium showed growth first at the top of the slant where the agar thickness was the slightest and with prolonged incubation progressed down the slant. It was proposed that the effect was due to a larger ratio of cells to agar at the thin end, and thus a larger effective inoculum size. To duplicate the situation seen in the agar slants under more controlled conditions, agar wedge plates were used. Plates of UB-M9 agar with various concentrations of Ni were poured on a slanted surface so the agar solidified in a wedge form, with a thickness from less than 0.1 to 6 mm. The plates were inoculated by spreading 0.1 ml/plate of a washed starter culture of a 10^{-2} dilution of it. The occurrence and position of growth on the plates was observed, measured, and photographed at intervals.

Fig. 34. Lag time (h) versus inoculum size on growth of A. marinus in UB-M9 medium of different Ni concentrations. Theoretical curve is drawn on basis of lag time doubling with each halving of inoculum size.


0 Ni
1 x 10-5 M Ni
2 x 10-5 M Ni
5 x 10-5 M Ni
1 x 10-4 M Ni
theoretical curve
Lag, h
720
640
560
480
400
320
240
160
80
0
0.2
0.6
1.0
1.4
1.8
2.2
2.6
Inoculum size, ml

The growth of A. marinus with an inoculum of 0.018 ml EQ (on a 100 ml agar basis, plates 20 ml agar per 100 mm square dish) on such a plate is shown in Fig. 35. Growth, as visible colonies, progressed from the thinnest (top) to thickest (bottom) parts of the plate as incubation time increased. The lag time before any visible colonies appeared was 20 h on the 5×10^{-5}M plate and 90 h on the 1×10^{-4}M Ni plate (Table 29). Data for the full set of Ni concentrations and the two inoculum sizes used is shown in Table 29. In view of the immediate growth at 1 and 2×10^{-4}M Ni with a 1.8 ml EQ inoculum, there was an appreciable shift in the range of Ni effect on agar as opposed to broth medium (compare Table 19). This might be due to the ability of this algal polysaccharide to act as a cation exchanger (Penniman, personal communication). For the 5×10^{-5} and 1×10^{-4}M Ni, 0.018 ml EQ inoculum treatments, the regression of growth distance versus incubation time was linear (Table 29), though the slopes were different. As the effective inoculum size (cells per unit volume of agar) varied with agar depth, a linear relationship between lag time of A. marinus and the ratio of inoculum to medium volume existed, confirming the occurrence of an inoculum dependent lag time.

Cell Adaptation to Ni

Although the bioassay and the inoculum size experiments indicated that conditioning of the medium was the most likely mechanism for the termination of Ni-induced lag of A. marinus in UB-M9 medium, the possibility existed that cell adaptation was the single mode of action, or both cell adaptation and medium

Fig. 35. UB-M9 medium agar wedge plates of A. marinus, 5 X 10^{-5}M Ni, 0.018 ml EQ inoculum after 65, 90, and 120 h incubation.

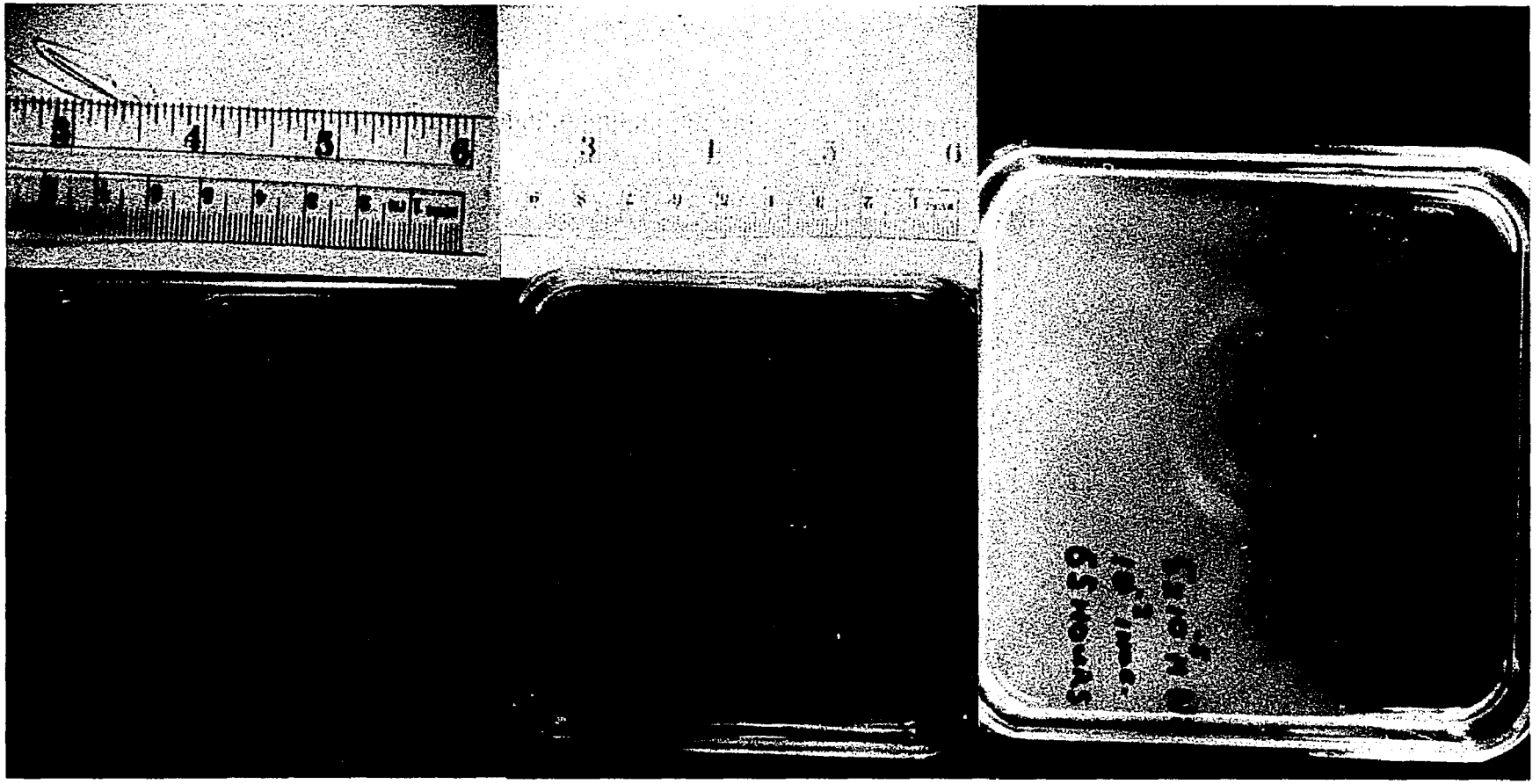


Table 29. UB-M9 medium agar wedge plates of A. marinus, growth at 20 h incubation.

Inoculum	Ni, M of agar	Growth
1.8 ml EQ	0 to 2 X 10^{-5}	equal growth throughout
	3, 5 X 10^{-5}	growth throughout, strongest at thin end
	1, 2 X 10^{-4}	no growth (growth throughout at 46 h)
0.018 ml EQ	0 to 3 X 10^{-5}	very weak growth throughout
	5 X 10^{-5}*	no growth
	1, 2 X 10^{-4}*	no growth

*Growth through 216 h below as mm from thin end of plate.

	Time, h							
Inoculum	20	45	65	90	120	144	168	216
5 X 10^{-5}[1]	0	25	35	60	100	-	-	-
1 X 10^{-4}[2]	0	0	0	0	15	30	40	50
2 X 10^{-4}	0	0	0	0	0	0	0	0

[1]linear regression data: r=0.994, m= 0.990, b= -22.1.
Significant at 99.9% level.

[2]linear regression data: r=0.975, m= 0.404, b= -32.7
Significant at 99 % level.

conditioning had a role in the lag phase phenomenon. If the lag phase was due to a cell adaptive response, either by a phenotypic or genotypic mechanism, cells grown in the presence of Ni should give a reduced lag time when used to inoculate fresh Ni containing UB-M9 medium compared to an inoculum not exposed to Ni.

The extent of cell adaptation to Ni was assayed by using A. marinus cells grown to late log or early stationary phase in UB-M9 medium containing 2×10^{-5} or 5×10^{-5}M Ni. Otherwise, the inoculum was handled exactly as was a standard 0 Ni-grown inoculum used as a control. For one of the experiments it was attempted to harvest cells by centrifugation in mid-lag phase from a 1500 ml volume of UB-M9 broth inoculated with 1.0 ml EQ (per 100 ml, total 15 ml). All inocula were washed and standardized turbidimetrically as for the standard inoculum. The different inocula were inoculated into replicate flasks from the same batch of medium with a range of Ni concentrations and the growth monitored.

The results of an experiment using 2×10^{-5}M Ni are shown in Table 30. The attempt to harvest cells from a lag phase culture failed, giving abnormally long lag times even at 0 Ni. The effective size of that inoculum was probably very small, perhaps because the centrifugation used to recover the cells also collected the $Ca_3(PO_4)_2$ precipitate from the large volume of medium. The precipitate would have interfered with the turbidimetric standardization of the inoculum leading to a smaller than intended effective inoculum size. Comparing the 0 and 2×10^{-5}M Ni grown inocula, at no Ni concentration

Table 30. *A. marinus* cell adaptation to Ni in UB-M9 medium (I). Lag time versus Ni for three inoculum treatments.

Treatment	Ni, M	Lag, h
No adaptation	0	c.10
	1×10^{-5}	20
	2×10^{-5}	35
	5×10^{-5}	75
	1×10^{-4}	140
2×10^{-5}M Mid lag phase cells	0	35
	1×10^{-5}	84
	2×10^{-5}	110
	5×10^{-5}	110
	1×10^{-4}	DNG
2×10^{-5}M Log phase Ni grown cells	0	12
	1×10^{-5}	25
	2×10^{-5}	33
	5×10^{-5}	104
	1×10^{-4}	190

did the Ni grown inoculum show any reduction of lag time. This experimental design was repeated using a higher (5×10^{-5} M) Ni concentration (Table 31). Again, there was little or no lag time reduction attributable to cell adaptation. The small apparent reductions of the Ni grown cells as compared to the 0 grown cells at 1×10^{-5}, 2×10^{-5} and 5×10^{-5} M Ni were well within the experimental error of replicate flasks in an UB-M9 growth curve set. Cell adaptation to Ni thus played no role in the A. marinus - Ni lag phase phenomenon.

From the results of the above three experiment sets, the conditioning bioassay, the inoculum size effect, and the cell adaptation, there was no evidence for a role of cell adaptation in the effect. Rather, medium conditioning occurred by a mechanism in which the effective concentration of Ni in the medium was reduced during the lag phase, so that by the end of the lag the Ni toxicity had been reduced below the threshhold concentration at which Ni inhibited cell division and growth.

The question of the actual mechanism of Ni detoxification has not yet been addressed. Possible mechanisms were uptake of Ni by cells of the inoculum, precipitation of the Ni as a highly insoluble form (such as a sulfide), and formation of organometallic compounds or complexes of Ni which were non-toxic as compared to the free metal ion.

Table 31. A. marinus cell adaptation to Ni in UB-M9 medium (II). Lag time versus Ni for two inoculum treatments.

Treatment	Ni, M	Lag, h
No adaptation	0	10
	1×10^{-5}	20
	2×10^{-5}	60
	5×10^{-5}	110
	1×10^{-4}	125
5×10^{-5}M Log phase Ni grown cells	0	15
	1×10^{-5}	17
	2×10^{-5}	40
	5×10^{-5}	100
	1×10^{-4}	160

Growth Effect of Chelating Agents

The effect of known chelating agents on the growth of A. marinus was studied to determine if such compounds could act in a manner similar to that of the proposed organometallic medium conditioning agents. Two classes of known, synthetic chelating compounds were studied. The first type was the iron-specific metal transport compounds, the siderophores (Neilands, 1974). These compounds have a widespread or universal role in bacterial iron transport. They also are the active agents of an inoculum-dependent lag phenomenon in B. subtilis (Lankford et al., 1966), which, though due to a nutrient deficiency (Fe starvation) rather than metal toxicity inhibition of cell division, bore several similarities to the lag phenomenon of A. marinus including the ability to detect the conditioning agent by bioassay of pregrown medium. The possibility was considered that the Ni-induced inoculum dependent lag of A. marinus in M9 medium was due to Ni interference with a siderophore-mediated Fe transport system.

A commercially available siderophore, the trihydroxamic acid, Desferal (Ciba), was examined over a range of concentrations known to act in either the reduction of inoculum-dependent lag or stimulation of the growth of siderophore auxotrophs. UB-M9 was prepared with 0 or 1 X 10^{-4}M Ni and Desferal concentrations from 0 to 1000 µg/liter (1000 µg/liter = 1.78 X 10^{-6}M). Flasks were inoculated with 1.0 ml EQ of a washed A. marinus inoculum and the growth monitored. Table 32 gives the growth parameters for all Desferal concentrations. There was a slight reduction in lag time at 5 to 100 µg/liter

Table 32. Effect of Desferal concentrations on the growth of A. marinus in UB-M9 medium with 0 and 1 X 10^{-4}M Ni.

Ni, M	Desferal µg/liter	Doubling time, h	OD_{MAX}	Lag, h
0	0	1.87	1.83	8
	1	1.96	1.90	8
	5	1.58	1.68	8
	10	2.75	2.15	8
	50	2.46	1.98	8
	100	1.98	1.80	8
	1000	1.51	1.66	8
1 X 10^{-4}	0	7.81	1.66	22
	1	6.43	1.54	20
	5	8.81	1.36	14
	10	10.8	1.70	14
	50	8.09	1.62	14
	100	5.77	1.48	14
	1000	4.97	2.16	22

Desferal concentration and 1 X 10^{-4}M Ni. However, those cultures had a slower growth rate. Similarly, the 0 Ni flasks indicated a slight increase in OD_{MAX} at 10 µg/liter Desferal. The Desferal effect was slight, perhaps within the range of random experimental error, occurring at about 10 µg/liter (1.78 X 10^{-7}M) and disappearing above or below that concentration. At that low a concentration the siderophore might have stimulated slightly the culture due to increased Fe availability. The lag of the culture was not dependent on siderophore concentration in either the presence or absence of Ni.

Other chelating agents studied were the non-specific compounds typified by the high metal-avidity synthetic chemical, EDTA, or the amino acid, histidine. Peptone, a protein digest containing free amino acids and short chain peptides giving a complex mixture of chelating compounds of varying metal avidities, also was examined. If medium conditioning was mediated by organometallic complexation, such chelating compounds would be useful models. Sets of UB-M9 with 0, 1 X 10^{-5}, 2 X 10^{-5}, 5 X 10^{-5}, and 1 X 10^{-4}M Ni were prepared with added concentrations of 2 X 10^{-5} or 1 X 10^{-4}M EDTA or histidine, 2 µg/ml peptone (Fisher) (equivalent to 1 X 10^{-5}M of 200 dalton amino acids as an approximation) or no additions (standard UB-M9). The flasks were inoculated with 1.0 ml EQ of a washed A. marinus inoculum and the growth monitored (Table 33).

EDTA gave rise to a quantitative suppression of the inhibitory effects of the Ni in a 1:1 molar ratio. Thus, with 2 X 10^{-5}M EDTA there was no lag at 1 or 2 X 10^{-5}M Ni and a lag

Table 33. The effect of different chelating agents on the growth parameters of A. marinus in UB-M9 of different Ni concentrations.

Chelator	Ni, M	Doubling time, h	OD_{MAX}	Lag, h
	0	2.92	1.96	8
UB-M9 (I)	1×10^{-5}	4.13	1.43	20
	2×10^{-5}	14.8	0.89	40
	5×10^{-5}	13.0	0.92	c.100
	1×10^{-4}	7.69	1.45	160
	0	2.96	1.69	8
UB-M9 (II)	1×10^{-5}	5.95	1.36	20
	2×10^{-5}	18.2	0.96	40
	5×10^{-5}	12.4	1.37	c.100
	1×10^{-4}	8.31	1.36	150
+2×10^{-5}M	0	2.55	2.39	8
histidine	1×10^{-5}	3.07	1.96	8
	2×10^{-5}	3.15	2.11	8
	5×10^{-5}	6.92	1.32	8
	1×10^{-4}	6.34	0.678	30
+1×10^{-4}M	0	2.57	2.44	8
histidine	1×10^{-5}	2.60	2.47	8
	2×10^{-5}	2.64	2.32	8
	5×10^{-5}	2.87	2.19	8
	1×10^{-4}	3.55	1.79	10

Table 33 Continued.

Chelator	Ni, M	Doubling time, h	OD_{MAX}	Lag, h
+2 X 10^{-5}M	0	4.17	1.53	10
EDTA	1 X 10^{-5}	4.15	1.49	10
	2 X 10^{-5}	2.72	1.39	8
	5 X 10^{-5}	17.6	1.52	60
	1 X 10^{-4}	9.67	2.16	c.100
+1 X 10^{-4}M	0	4.39	1.99	12
EDTA	1 X 10^{-5}	5.34	1.53	12
	2 X 10^{-5}	4.71	1.68	12
	5 X 10^{-5}	4.59	1.54	12
	1 X 10^{-4}	3.14	1.33	8
+2 µg/ml	0	2.72	1.83	8
peptone	1 X 10^{-5}	4.87	1.13	12
	2 X 10^{-5}	8.04	0.659	20
	5 X 10^{-5}	22.3	1.58	45
	1 X 10^{-4}	17.6	1.66	c.110

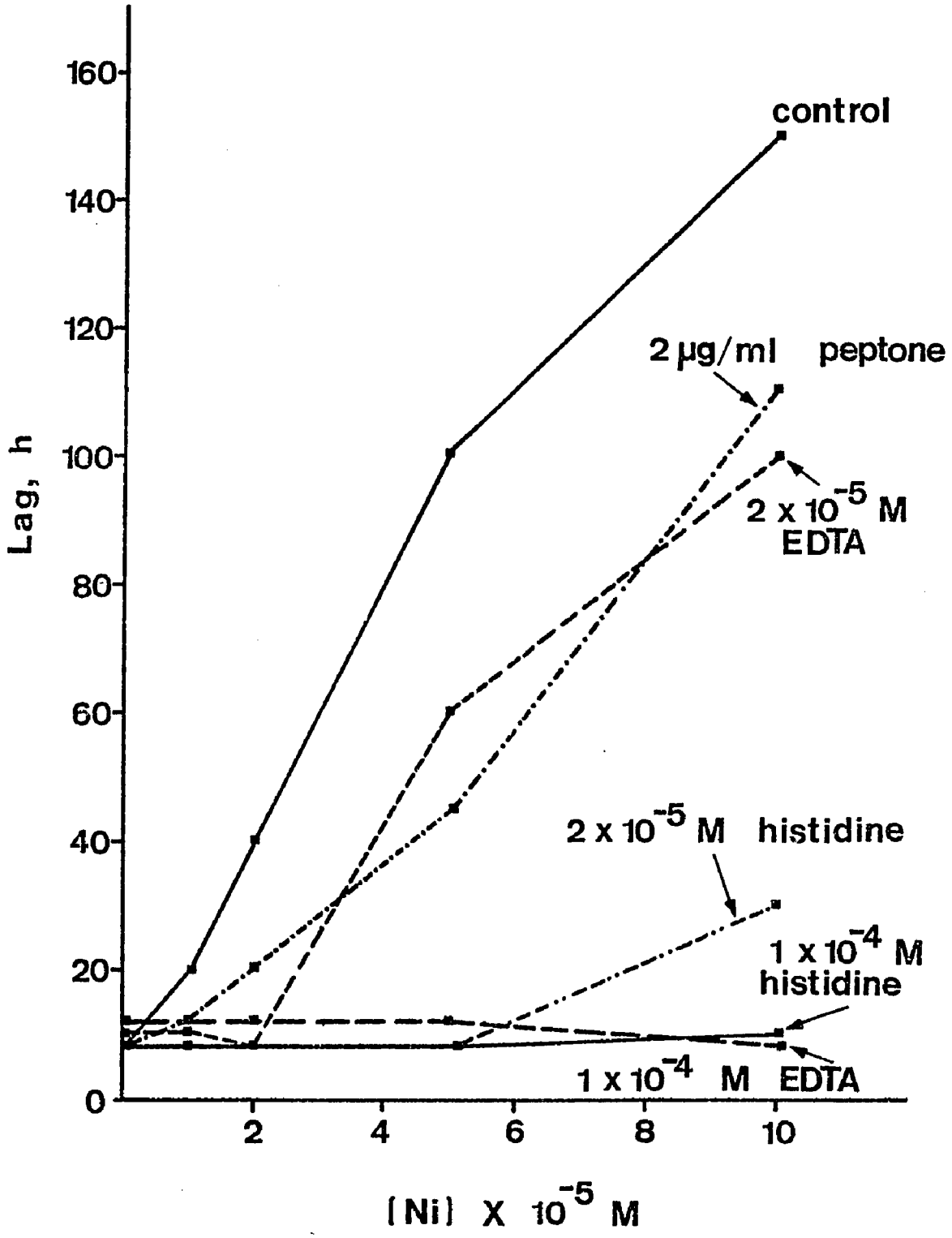
at 5 X 10^{-5}M Ni which was intermediate between the 2 and 5 X 10^{-5}M Ni control lag phases. This would be expected if the effective Ni concentration in that flask was: 5 X 10^{-5} - 2 X 10^{-5} = 3 X 10^{-5}M Ni. In both EDTA concentrations the doubling time was lowest at an equimolar chelator:Ni ratio. This growth stimulation might indicate that EDTA was making essential nutrient cations unavailable to the cells when EDTA was not as concentrated as Ni.

The pattern with histidine was more complex. Thus, at a histidine concentration of 2 X 10^{-5}M, there was no inhibition of the culture up to a Ni concentration of between 5 X 10^{-5} and 1 X 10^{-4}M Ni. This might indicate that each amino acid molecule was acting as a chelating agent for two or three Ni ions. The slight inhibition noted with EDTA at excess chelating agent:Ni ratios was not seen with histidine.

The situation with 2 µg/ml peptone was less distinct. At 1 X 10^{-5}M Ni there was a 12 h lag compared to the 0 Ni flask of UB-M9 with 2 µg/ml peptone (8 h) but the lag observed was less than the 1 X 10^{-5}M Ni flasks of the control sets (20 h). There was a similar reduction of lag times at the higher Ni concentrations, the magnitude of the reduction equivalent to a Ni concentration decrease of about 1 X 10^{-5}M for each Ni concentration.

The plot of the Ni concentration versus lag time (Fig. 36) showed the typical linear relationship for the control sets. EDTA, 2 X 10^{-5}M, and 2 µg/ml peptone illustrated similar linear responses of similar slope to the control, but with the lines displaced along the x-axis, as might be expected

Fig. 36. Lag time (h) versus Ni concentration (X 10^{-5}M) for A. marinus grown in UB-M9 medium with different chelating agents (I).


160
140
120
100
80
60
40
20
0
Lag, h
2
4
6
8
10
[Ni] X 10^-5 M
control
2 µg/ml peptone
2 x 10^-5 M EDTA
2 x 10^-5 M histidine
1 x 10^-4 M histidine
1 x 10^-4 M EDTA

from a quantitative reduction in effective Ni concentration. However, the peptone treatment intersected the axis at about the same points as the control due to curving of the plot near the origin. Thus, the effect of peptone on A. marinus growth was defined less sharply than that of EDTA. Histidine (1 X 10^{-4}M) and 1 X 10^{-4}M EDTA treatments showed no increase in lag time even at 1 X 10^{-4}M Ni. This would be expected if the chelating agents were quantitatively sequestering the Ni. The situation with 2 X 10^{-5}M histidine was intermediate between the above two cases as would be expected if each histidine molecule was complexing more than one Ni ion.

The experimental design above was repeated giving essentially identical results (Fig. 37). Known metal complexing agents acted stoichiometrically to reduce the lag time response of A. marinus in UB-M9 at a normally inhibitory Ni concentration. These known chelating agents behaved in a similar manner to that of the proposed medium conditioning agents.

Differential Pulse Polarography of Known Chelating Agents

The pulse polarographic technique (DPP) was used also to examine the effect of changes in metal speciation due to known chelating agents on the electrochemical forms of the metal. Duplicate flasks to those used for the growth studies above were prepared simultaneously with the growth media and analyzed by DPP. The control polarograms (Fig. 38) showed the height of the Ni peak rising proportionally to the Ni concentration added. The data for peak height and peak potential

Fig. 37. Lag time (h) versus Ni concentration (X 10^{-5}M) for *A. marinus* grown in UB-M9 medium with different chelating agents (II).

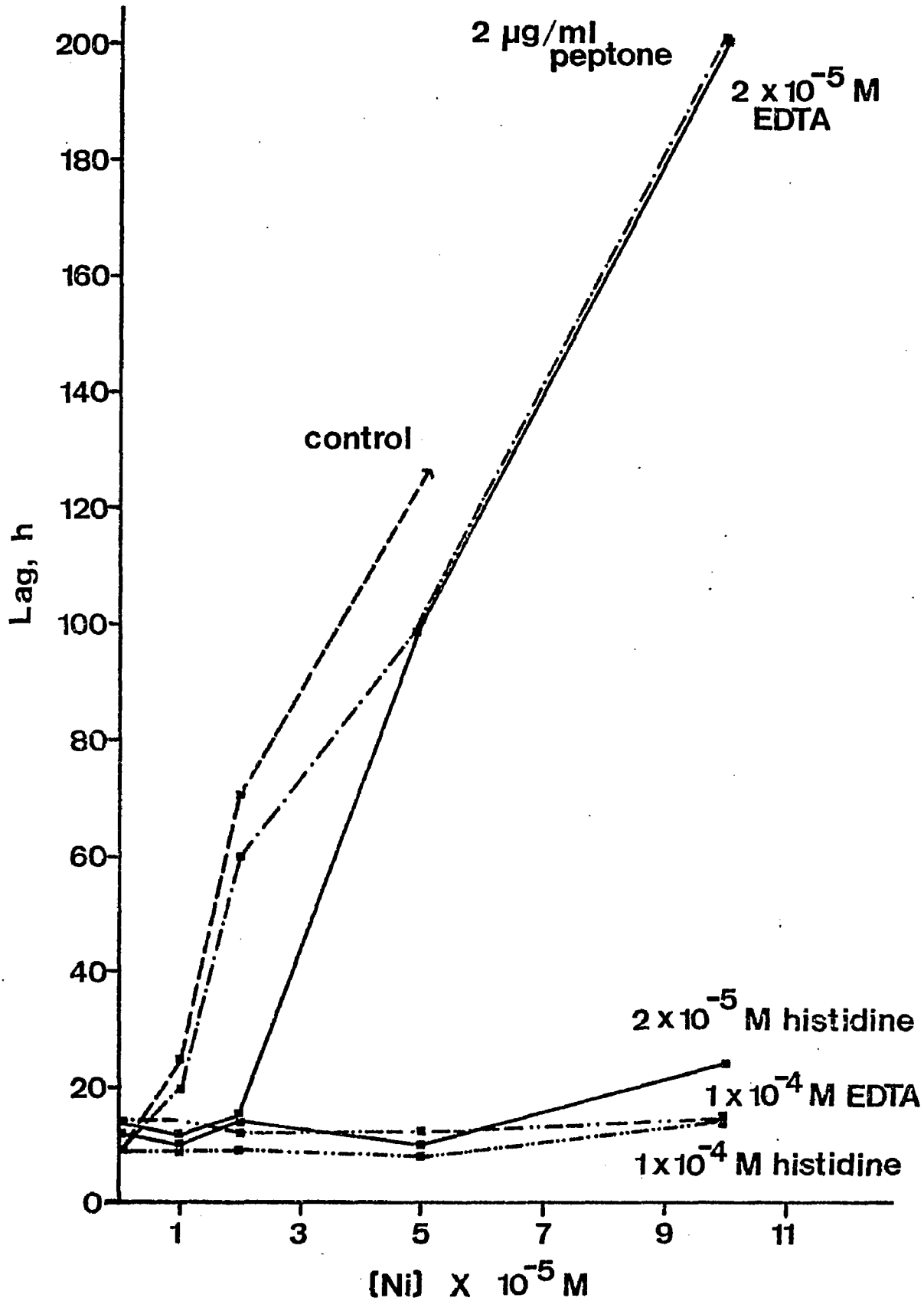

2 µg/ml peptone
2 x 10⁻⁵ M EDTA
control
2 x 10⁻⁵ M histidine
1 x 10⁻⁴ M EDTA
1 x 10⁻⁴ M histidine
Lag, h
200
180
160
140
120
100
80
60
40
20
0
1
3
5
7
9
11
[Ni] X 10⁻⁵ M

Fig. 38. DPP plot of UB-M9 with no chelator added at 5 different Ni concentrations: A. 0 Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v, 2 mv/sec scan rate, 1 sec drop time, 2 μA full scale sensitivity.

Signal amplitude (2μA full scale)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)
A
B
C
D
E

for each treatment is presented in Table 34. The Ni peak in the control was essentially constant in potential, rising in height proportionately to Ni concentration (r=0.996 at 2.19 X 10^6 mm/M). This regression was used to calculate the "Ni measured" for the remaining polarograms. Peptone (Fig. 43) gave a Ni peak which also rose in direct proportion to Ni, if the effective chelating agent concentration estimated at 1 X 10^{-5}M was subtracted from the Ni added to give an effective free Ni concentration. However, unlike the strong chelating agents, there was an appreciable peak present at 1 X 10^{-5}M Ni added. Indeed, the measured Ni concentration (from the O chelator regression) was higher at 1 and 2 X 10^{-5}M Ni with 2 ppm (mg/liter) peptone than in the UB-M9 medium control.

The polarograms of EDTA-containing UB-M9 medium (Figs. 39, 40) showed the pattern expected from the growth response. In the presence of 2 X 10^{-5}M EDTA there was no Ni peak visible until 2 X 10^{-5}M Ni had been exceeded. Above 2 X 10^{-5}M Ni the peak height rose proportionately (r=0.997) with the Ni concentration minus the chelator concentration (Table 34). At 1 X 10^{-4}M EDTA there was no Ni peak even at 1 X 10^{-4}M Ni. In the specific case of EDTA, only free Ni ion was toxic to the bacterium, Ni in the form of EDTA-Ni complexes was non-toxic.

The situation with histidine was more complicated. The polarograms for the two histidine concentrations studied, Figs. 41 and 42, indicated multiple peaks. At O Ni in the 5 X 10^{-5}M histidine medium, the baseline was essentially identical to the control medium, but when 1 X 10^{-5}M Ni was

Table 34. Effect of chelating agents on the polarographic response of Ni in UB-M9 medium.

Chelator	Ni, M	Chelator, M	Ni - Ch	peak[a] height[b]	potential	/Ni/, meas.[d]M
A. none	0	0	0	0	-	-
r=0.996[c]	1×10^{-5}	0	1×10^{-5}	10	-1.10	7.11×10^{-6}
m=2.19 X 10^6mm/M[c]	2×10^{-5}	0	2×10^{-5}	31	-1.09	1.67×10^{-5}
b=-5.60 mm[c]	5×10^{-5}	0	5×10^{-5}	116	-1.09	5.54×10^{-5}
	1×10^{-4}	0	1×10^{-4}	c.210	-1.09	9.82×10^{-5}
B. 2×10^{-5}M EDTA	0	2×10^{-5}	-2×10^{-5}	0	-	-
r=0.9997	1×10^{-5}	2×10^{-5}	-1×10^{-5}	0	-	-
m=1.77 X 10^6mm/M	2×10^{-5}	2×10^{-5}	0	1	-	3.01×10^{-6}
b=-1.73	5×10^{-5}	2×10^{-5}	3×10^{-5}	51	-1.08	2.58×10^{-5}
	1×10^{-4}	2×10^{-5}	8×10^{-5}	142	-1.08	6.73×10^{-5}
C. 1×10^{-4}M EDTA	0	1×10^{-4}	-1×10^{-4}	0	-	-
	1×10^{-5}	1×10^{-4}	-9×10^{-5}	0	-	-
	2×10^{-5}	1×10^{-4}	-8×10^{-5}	0	-	-
	5×10^{-5}	1×10^{-4}	-5×10^{-5}	0	-	-
	1×10^{-4}	1×10^{-4}	0	4	-	4.37×10^{-6}

Table 34 Continued.

Chelator	Ni, M	Chelator, M	Ni - Ch	peak[a] height[b]	potential	[Ni], meas.M
D. 2×10^{-5}M histidine	0	2×10^{-5}	-2×10^{-5}	0	-	-
	1×10^{-5}	2×10^{-5}	-1×10^{-5}	0	-	-
r=0.966	2×10^{-5}	2×10^{-5}	0	0	-	-
m=6.11 $\times 10^{-5}$mmM	5×10^{-5}	2×10^{-5}	3×10^{-5}	6	-1.07	5.29×10^{-6}
b=-1.73	1×10^{-4}	2×10^{-5}	8×10^{-5}	47	-1.08	2.40×10^{-5}
E. 1×10^{-4}M histidine	0	1×10^{-4}	-1×10^{-4}	0	-	-
	1×10^{-5}	1×10^{-4}	-9×10^{-5}	0	-	-
	2×10^{-5}	1×10^{-4}	-8×10^{-5}	0	-	-
	5×10^{-5}	1×10^{-4}	-5×10^{-5}	0	-	-
	1×10^{-4}	1×10^{-4}	0	0	-	-
F. 2 µg/ml peptone[e]	0	1×10^{-5}	-1×10^{-5}	0	-	-
r=0.995	1×10^{-5}	1×10^{-5}	0	15	-1.10	9.39×10^{-6}
m=2.19 $\times 10^{6}$mmM	2×10^{-5}	1×10^{-5}	1×10^{-5}	38	-1.10	1.99×10^{-5}
b=17.6	5×10^{-5}	1×10^{-5}	4×10^{-5}	86	-1.08	4.17×10^{-5}
	1×10^{-4}	1×10^{-5}	9×10^{-5}	c.170	-1.09	8.00×10^{-5}

Table 34 Continued.

[a]"free Ni" peak -1.05 to -1.15 v band.

[b]measured by tangent baseline method.

[c]regression of peak height versus $[Ni] - [chelator]$.

[d]$[N]$ measured by applying set A regression to measured peak height.

[e]considered equivalent to 1 X 10^{-5}M of 200 mw amino acid.

Fig. 39. DPP plot of UB-M9 with 2 X 10^{-5}M EDTA added to 5 different Ni concentrations: A. 0 M Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v, 2 μA full scale sensitivity, 1 sec drop time, 2 mv/sec scan rate. Bl. indicates tangential baseline used for peak height determination.

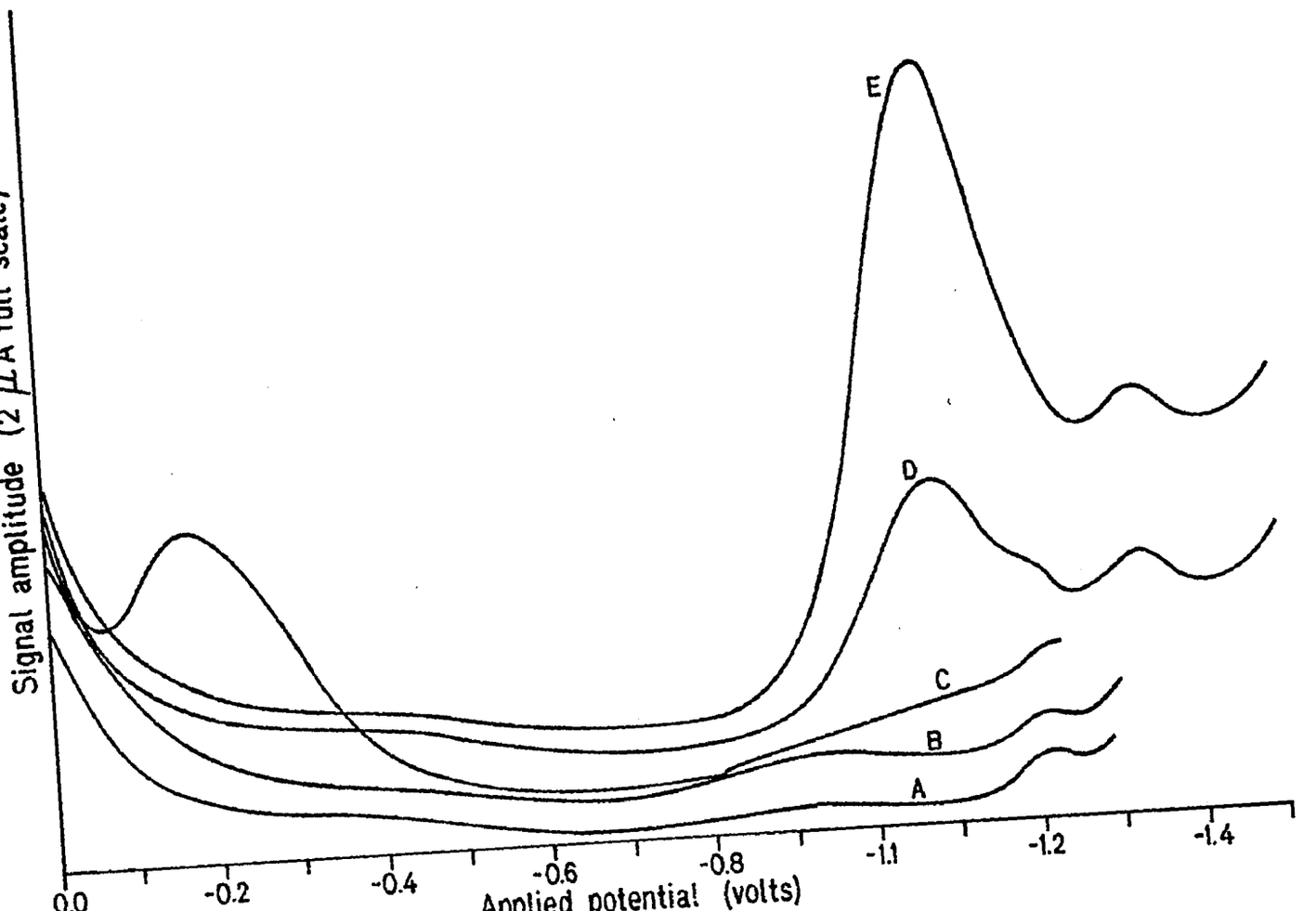

Signal amplitude (2 μA full scale)
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.1
-1.2
-1.4
Applied potential (volts)

Fig. 40. DPP plot of UB-M9 with 1 X 10^{-4}M EDTA added to 5 different Ni concentrations: A. 0 Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v, 2 μA full scale sensitivity, 1 sec drop time, 2 mv/sec scan rate.

Signal amplitude (2μA full scale)
E
C
D
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

Fig. 41. DPP plot of UB-M9 with 2 X 10^{-5}M histidine added to 5 different Ni concentrations: A. 0 Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v, 2 μA full scale sensitivity, 1 sec drop time, 2 mv/sec scan rate. Bl. indicates tangential baseline used for peak height determination.

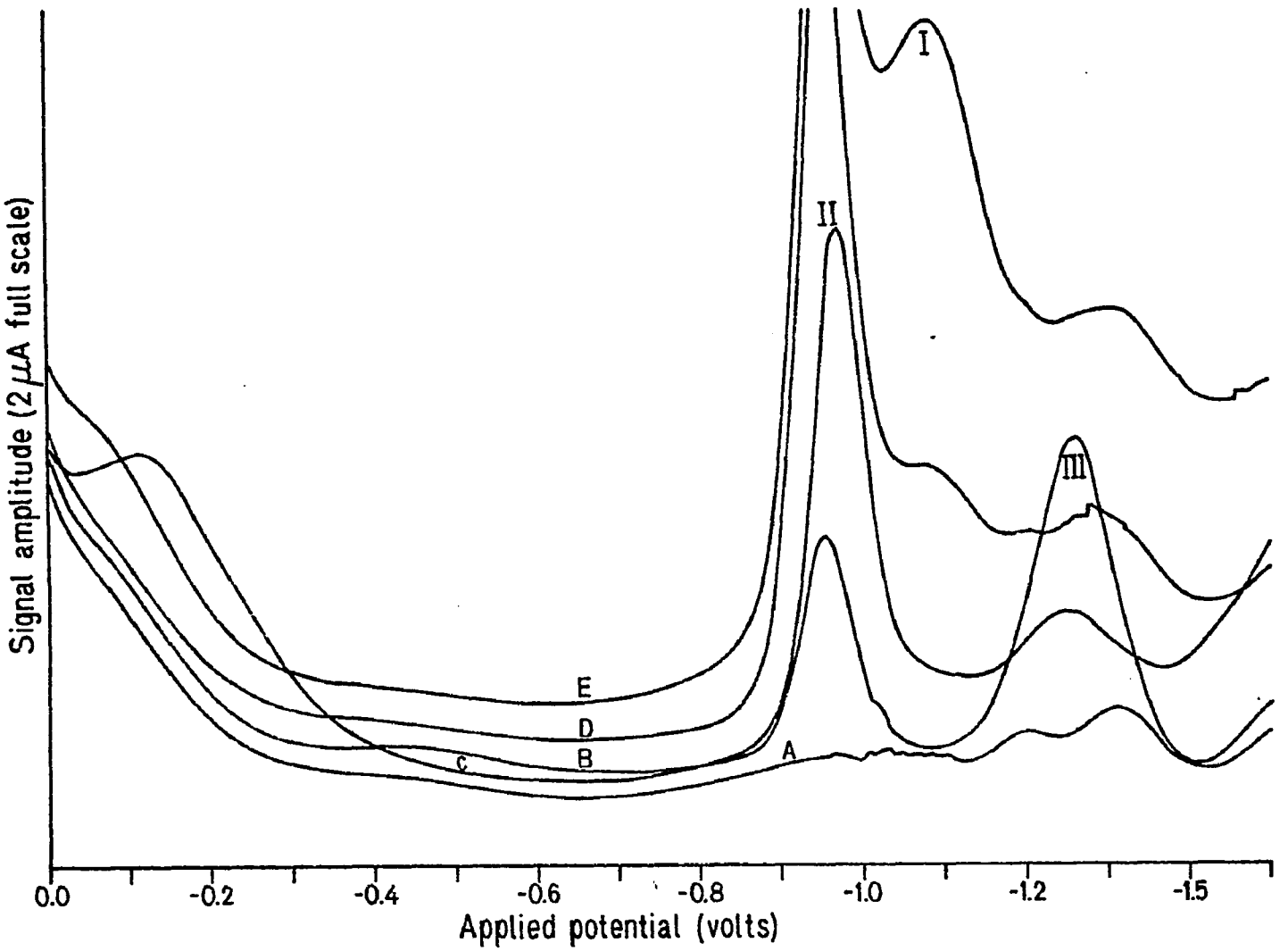
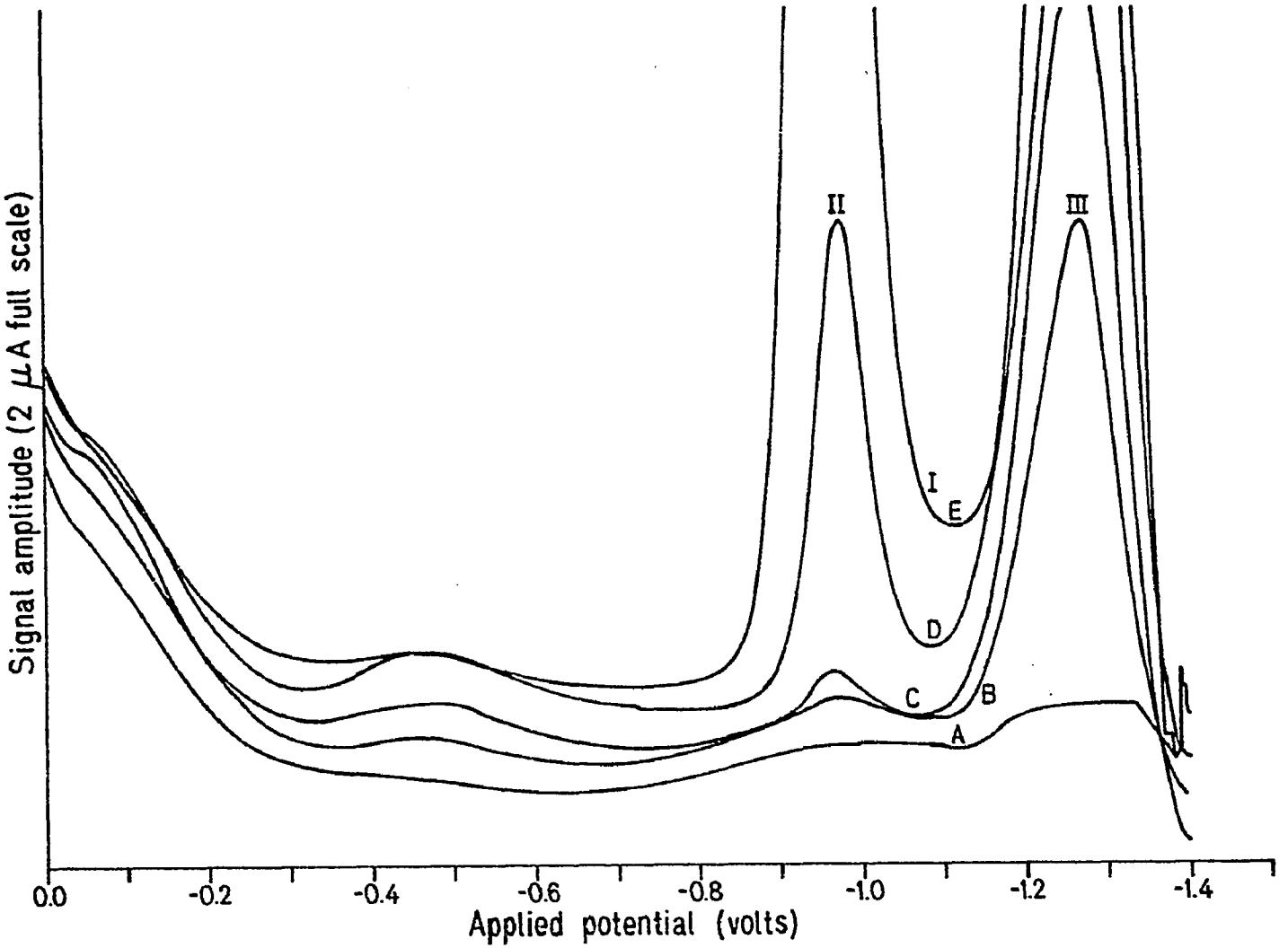

Signal amplitude (2 μA full scale)
I
II
III
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.5
Applied potential (volts)

Fig. 42. DPP plot of UB-M9 with 1 X 10^{-4}M histidine added to 5 different Ni concentrations: A. 0 Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v range, 2 μA full scale sensitivity, 1 sec drop time, 2 mv/sec scan rate.


Signal amplitude (2 μA full scale)
II
III
I
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

added two peaks appeared (-0.95 and -1.26 v). As the Ni concentration increased, the -1.26 v peak rapidly decreased, while the lower peak increased in size. A peak at the free Ni potential did not appear until the Ni concentration exceeded 5×10^{-5}M. In the 1×10^{-4}M histidine (Fig. 42) the "free" Ni peak never appeared. The -1.26 v peak increased in size up to 5×10^{-5}M Ni, decreasing again at 1×10^{-4}M Ni. This peak was dominant at chelating agent: Ni ratios of greater than 1. The low potential peak did not appear until 2×10^{-5}M Ni, and only became prominent above that Ni concentration. Evidently these two peaks were due to the interaction of the metal with the amino acid as they were absent in the medium with the chelator but without added Ni. However, it was unclear whether these peaks represented the reduction of complexed forms of the metal or reductions of the organic compound which were potentiated catalytically by the metal ions. The fact that these peaks were much greater in magnitude than the Ni peak in the control argued for the latter interpretation.

The polarographic pattern of UB-M9 medium containing 2 µg/ml peptone (Fig. 43) was qualitatively similar to the control polarograms. Resolution of that data (Table 34f) showed the Ni peak to rise proportionately with the added Ni concentration without any threshhold effects as were seen with EDTA and histidine. The measured Ni concentration was equal to the added Ni at 1 and 2×10^{-5}M Ni added, but was 20 % lower than the added concentration at 5 and 10×10^{-5}M. This was interpreted as indicating a small complexing capacity.

Fig. 43. DPP plot of UB-M9 with 2 µg/ml peptone added to 5 different Ni concentrations: A. 0 Ni. B. 1 X 10^{-5}M Ni. C. 2 X 10^{-5}M Ni. D. 5 X 10^{-5}M Ni. E. 1 X 10^{-4}M Ni. Potential scan 0.0 to -1.5 v range, 2 µA full scale sensitivity, 1 sec drop time, 2 mv/sec scan rate. Bl. indicates tangential baseline used for peak height determination.

Signal amplitude (2 μA full scale)
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

The polarographic phenomenon was studied further in a simplified system to determine if interaction with medium constituents or the autoclaving step of UB-M9 preparation had an effect on the metal speciation. Experiments in a 0.4 M KCl ionic matrix in which either a fixed concentration of the chelating agent was titrated with graded concentrations of Ni, or the converse experiment, a fixed concentration of Ni was titrated by graded concentrations of chelating agents. A 50 ml aliquot of the 0.4 M KCl was used in the polarographic cell, and the metal and chelating compound added as aliquants (10 to 100 µl) of sufficiently concentrated solutions that there was little volume change. The sample was mixed during the normal instrument 10 min deaeration step, and the polarogram run after each addition. In the case of EDTA, the results (not shown) completely confirmed the data from the above experiments. Both titrating the metal with the chelator, and vice versa, the height of the Ni peak was directly proportional to the $/\underline{\text{Ni}}/ - /\underline{\text{EDTA}}/$.

The histidine experiments gave a pattern distinctly different from that in the UB-M9 medium. The titration of 5×10^{-5}M histidine by Ni is shown in Fig. 44 (compare to Fig. 41). Here there were only two peaks, one of which occurred at -1.11 v, a potential in the range of "free" Ni. The second peak, at -0.94 v might be identical with the lower potential peak seen in histidine UB-M9 medium. The -1.26 v peak was not observed in this system. An appreciable "free" Ni peak was evident even at a histidine:Ni excess of 5:1.

Fig. 44. Polarographic effect of 5 X 10^{-5}M histidine titrated by Ni in 0.4 M KCl supporting electrolyte. A. 0.4 M KCl. B. 0.4 ml KCl + 5 X 10^{-5}M his. C. 1 X 10^{-5}M Ni + 0.4 M KCl + 5 X 10^{-5}M his. D. 0.4 M KCl + 5 X 10^{-5}M his + 2 X 10^{-5}M Ni. E. 0.4 M KCl + 5 X 10^{-5}M his + 5 X 10^{-5}M Ni. Scan range 0.0 to -1.5 v, 1 drop/sec, 2 mv/sec scan rate, 2 μA full scale sensitivity.

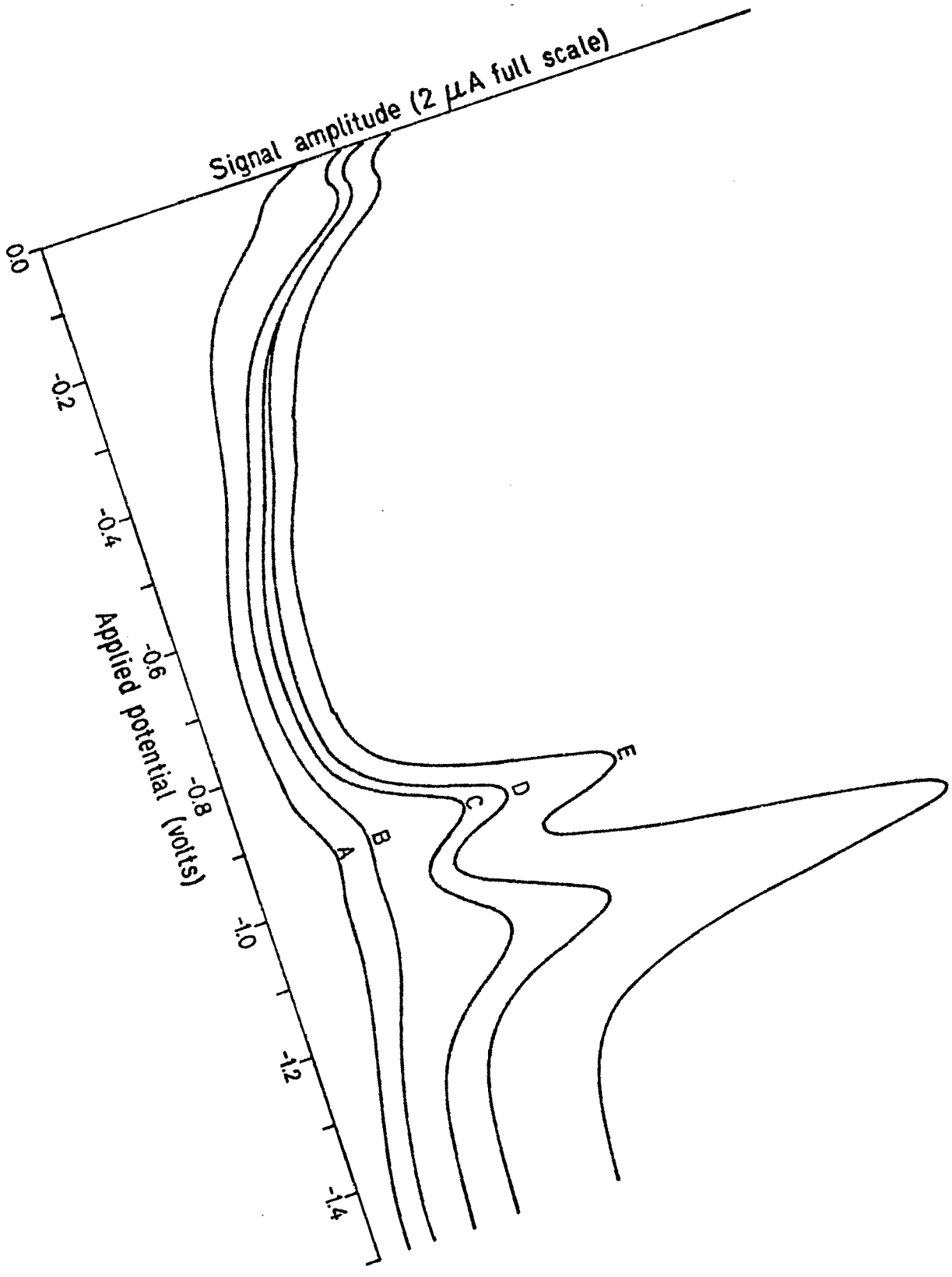

Signal amplitude (2 μA full scale)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)
A
B
C
D
E

Fig. 45 shows the converse experiment, 5 X 10^{-5}M Ni titrated by histidine. The peak at potential -1.11 v decreased only slightly in size as histidine concentration increased, the -0.94 v peak appeared as soon as histidine was added though at a potential higher than that at which it eventually stabilized. Again the third peak seen in UB-M9 was never observed. Apparently, the heat of autoclaving caused the formation of complex species which did not occur upon the initial mixing of the metal and amino acid. This data is presented as peak heights and potentials for both observed peaks in Table 35. The change in the size of the Ni peak with the concentration of chelating compound was much less direct than was seen in the autoclaved medium. Even at histidine molarities greater than the Ni molarity, the Ni peak was greater than 80 % of the peak in the absence of Ni. Evidently autoclaving, as was the case in the UB-M9 medium histidine experiment, was required for the formation of stable complexes of histidine-Ni. The complex pattern of polarographic peaks in the autoclaved medium with histidine might relate to the complex pattern of multiple overlapping peaks which was seen above with 2216E LN. If each of the amino acids present in the peptone based medium gave a characteristic pattern of multiple peaks, the summation of these might give a pattern as that in the complex medium.

Fig. 45. Polarographic effect of 5 X 10^{-5}M Ni titrated by histidine in 0.4 M KCl. A. 0.5 M KCl. B. 0.4 M KCl + 5 X 10^{-5}M Ni. C. 0.4 M KCl + 5 X 10^{-5}M Ni + 1 X 10^{-5}M his. D. 0.4 M KCl + 5 X 10^{-5}M Ni + 2 X 10^{-5}M his. E. 0.4 M KCl + 5 X 10^{-5}M Ni + 4 X 10^{-5}M his. F. 0.4 M KCl + 5 X 10^{-5}M Ni + 6 X 10^{-5}M his. G. 0.4 M KCl + 5 X 10^{-5}M Ni + 1 X 10^{-4}M his. Scan range 0.0 to -1.5 v, 1 drop/sec, 2 mv/sec scan rate, 2 μA full scale sensitivity.

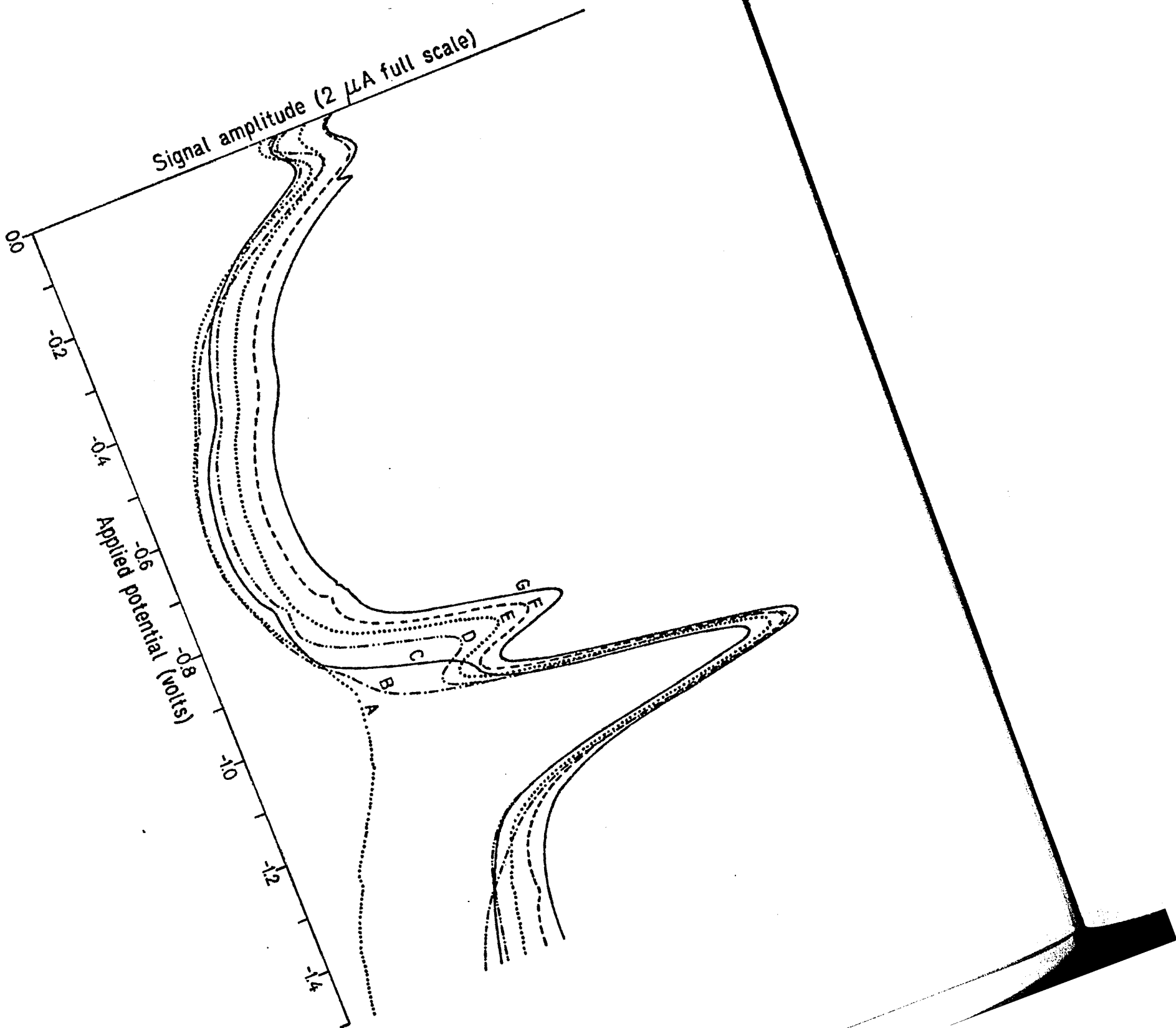

Signal amplitude (2 μA full scale)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)
A
B
C
D
E
F
G

Table 35. Quantitation of polarograms of histidine titrated by Ni (II) and vice versa (I) (Figs. 44-45) for the "free Ni" peak (-1.12v) and the secondary peak (-0.93 v) by both peak height and potential.

I.

			Ni peak		2^{o} peak	
Treatment	Ni, M	His, M	peak h[c]	potential	peak h	potential
Background[a]	0	0	0	-[b]	0	-[b]
+ 5 X 10^{-5}M Ni	5 X 10^{-5}	0	187	-1.12	0	-
+ 1 X 10^{-5}M his	5 X 10^{-5}	1 X 10^{-5}	163	-1.11	10	-0.99
+ 2 X 10^{-5}M his	5 X 10^{-5}	2 X 10^{-5}	158	-1.11	30	-0.95
+ 4 X 10^{-5}M his	5 X 10^{-5}	4 X 10^{-5}	164	-1.11	41	-0.94
+ 6 X 10^{-5}M his	5 X 10^{-5}	6 X 10^{-5}	152	-1.11	45	-0.93
+ 1 X 10^{-4}M his	5 X 10^{-5}	1 X 10^{-4}	153	-1.11	52	-0.93
+ 18 h equilibrium	5 X 10^{-5}	1 X 10^{-4}	137	-1.12	70	-0.94

[a]Background = 0.4 M KCl

[b]A single peak was impossible to read.

[c]peak height quantitation technique.

Table 35 Continued.

II

Treatment	Ni, M	His, M	Ni peak		2° peak	
			peak h	potential	peak h	potential
Background	0	0	2	-1.12	6	-
+ 5 X 10^{-5}M his	0	5 X 10^{-5}	4	-1.12	6	-
+ 1 X 10^{-5}M Ni	1 X 10^{-5}	5 X 10^{-5}	27	-1.11	37	-0.93
+ 2 X 10^{-5}M Ni	2 X 10^{-5}	5 X 10^{-5}	51	-	35	-0.93
+ 5 X 10^{-5}M Ni	5 X 10^{-5}	5 X 10^{-5}	139	-	47	-0.93

Physico-chemical Detection of Metal Binding Compounds

UV Spectroscopy

The occurrence of medium conditioning by A. marinus during lag phase, and the demonstration of the ability of known chelating agents (histidine and EDTA) to have a functionally identical role to an extracellular medium conditioning agent, led to an attempt to detect, isolate, and purify such extracellular metal binding agent(s) by physical and chemical techniques. The first physical technique examined was that of UV spectroscopy.

Many biologically significant molecules have prominent and characteristic absorption bands in the ultraviolet range of the electromagnetic spectrum. Familiar examples are the 280 nm absorbance band due to protein and the 260 nm absorbance characteristic of nucleic acids. The release of organic compounds into the medium during the lag and growth phases of A. marinus was monitored by scanning ultraviolet spectroscopy in the 320 to 190 nm wave band. A growth series of UB-M9 medium was prepared with added Ni concentrations of 0, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}, 5×10^{-5}, 1×10^{-4}M Ni. Flasks were inoculated with 1.0 ml EQ of washed A. marinus cells. At periods during the lag and growth of the cultures sample aliquots were taken, the growth as OD measured, the cells removed by filtration through 0.45-μm Millipore membranes and the UV absorbance scanned from 320 to 190 nm in matched quartz cuvettes in a Beckman DB-G dual-beam spectrophotometer with a

10" chart recorder. Samples were blanked against uninoculated UB-M9 medium. In all spectrograms there was a machine caused artifact peak at a wavelength of 210 to 200 nm.

Of the six Ni concentrations studied, three (0, 2 X 10^{-5}, and 1 X 10^{-4}M) were selected as representative. The growth of those cultures is plotted in Fig. 46. The pattern of growth was similar to that previously seen in A. marinus. Examination of the 0 Ni spectra (Fig. 47) showed the absence of any absorbance in the initial medium (other than baseline noise). Very rapidly during the lag phase (as early as 1 h incubation) a peak in the 220 nm region appeared. That peak continued to increase through the growth phase (36 and 48 h) and into stationary phase (72 h and later). During late log and early stationary phase a second peak appeared in the 260-280 nm range perhaps due to cellular release of proteins or nucleic acids. The pattern with 2 X 10^{-5}M Ni (Fig. 48) was essentially identical at comparable points in the growth curve. Again during the lag phase (0 to 48 h) the 215 nm peak became prominent and the 260-280 nm peak appeared only during growth. The same UV pattern was seen with A. marinus grown in 1 X 10^{-4}M Ni UB-M9 (Fig. 49).

As the 260-280 nm peak was absent during the lag phase, it was doubtful that those components participated in medium conditioning. The prominence of the 215 nm peak during the lag phase and its time dependent growth during the lag phase match features of the proposed medium conditioning agent. However, there were no differences in the appearance or size of the 215 nm peak in relationship to the Ni content of the

Fig. 46. Growth as OD of A. marinus in UB-M9 medium at 25°C with various Ni concentrations.

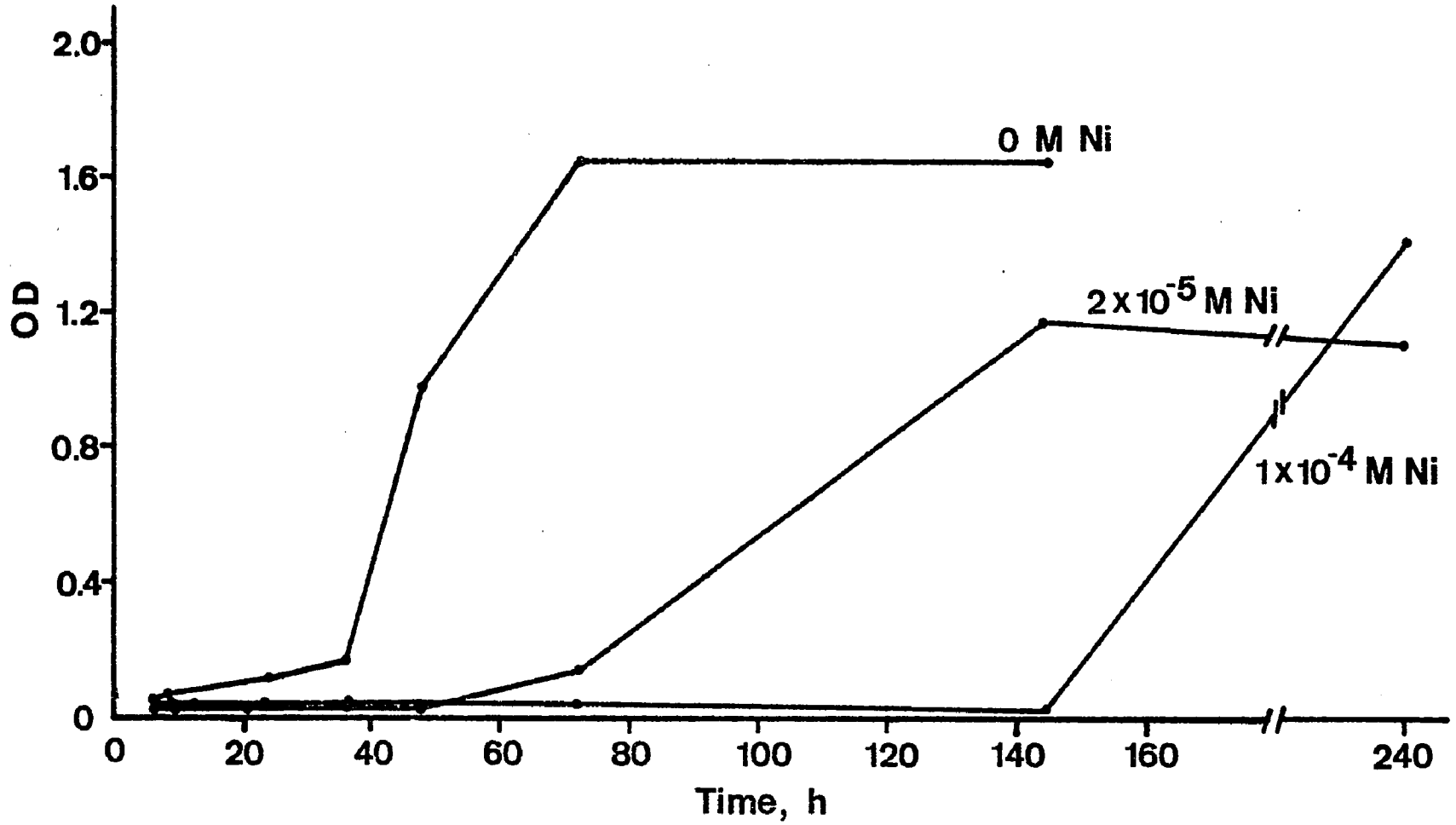

2.0
1.6
1.2
0.8
0.4
0
OD
0 M Ni
2x10-5 M Ni
1x10-4 M Ni
0
20
40
60
80
100
120
140
160
240
Time, h

Fig. 47. Ultraviolet spectroscopy of UB-M9 medium inoculated with A. marinus. Samples of medium were taken, filtered to remove light scattering material and UV scanned from 340 to 190 nm, blanked against uninoculated medium.

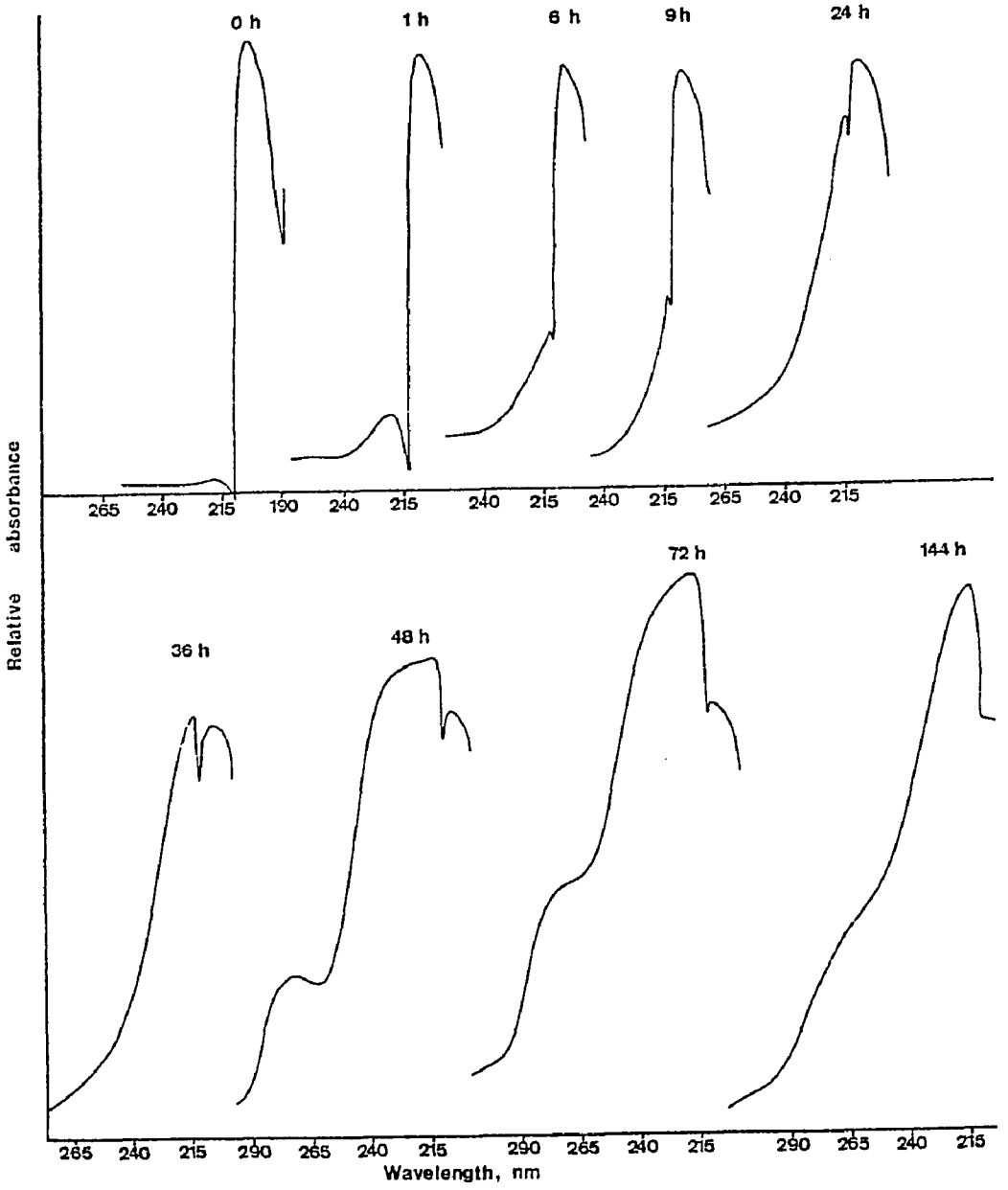

0 h
1 h
6 h
9 h
24 h
36 h
48 h
72 h
144 h
Relative absorbance
265 240 215 190 240 215 240 215 240 215 265 240 215
265 240 215 290 265 240 215 290 265 240 215 290 265 240 215
Wavelength, nm

Fig. 48. Ultraviolet spectroscopy of UB-M9 medium, 2 X 10^{-5}M Ni incubated with A. marinus. Samples of medium were taken, filtered to remove light scattering material and UV scanned from 340 to 190 nm, blanked against uninoculated medium.

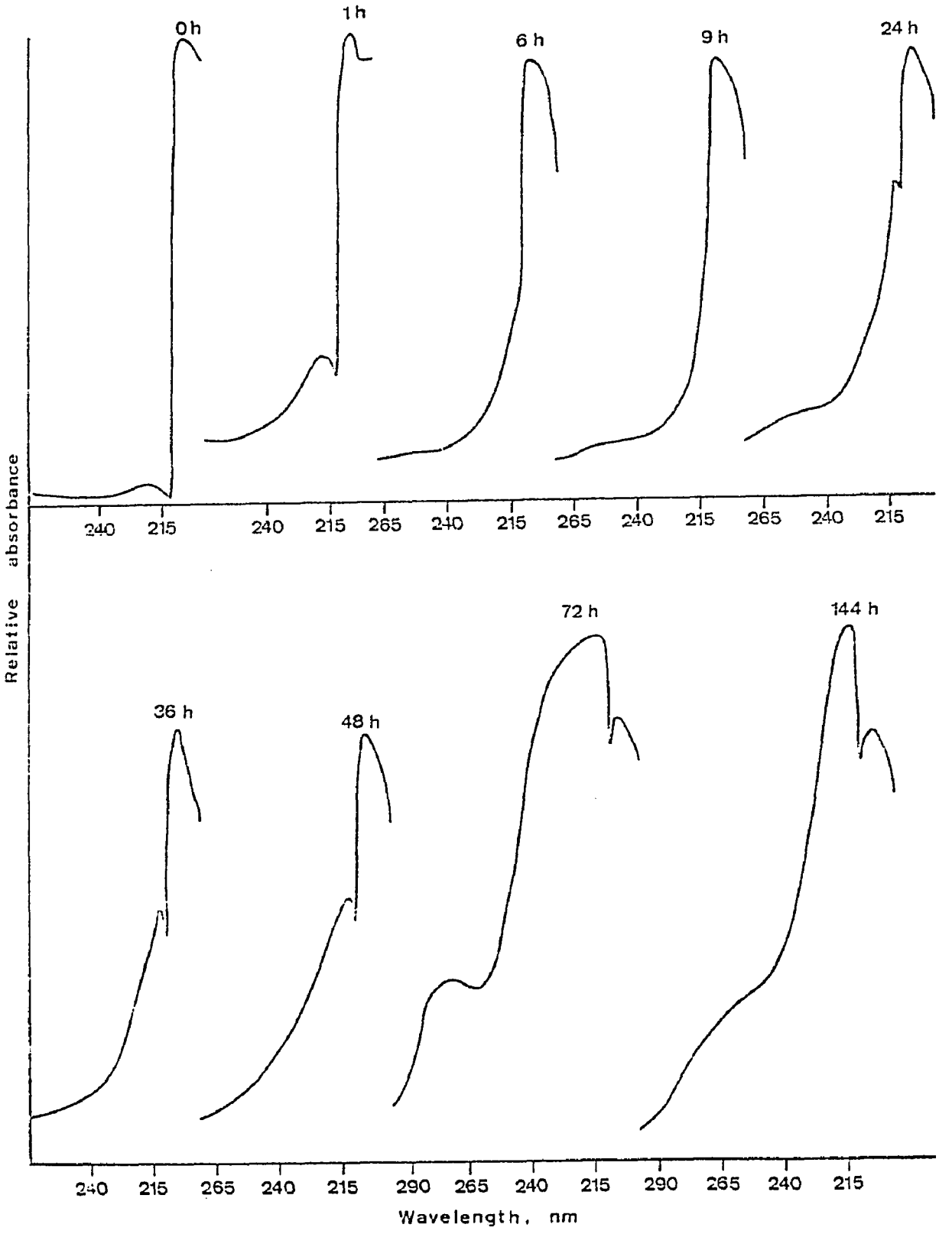
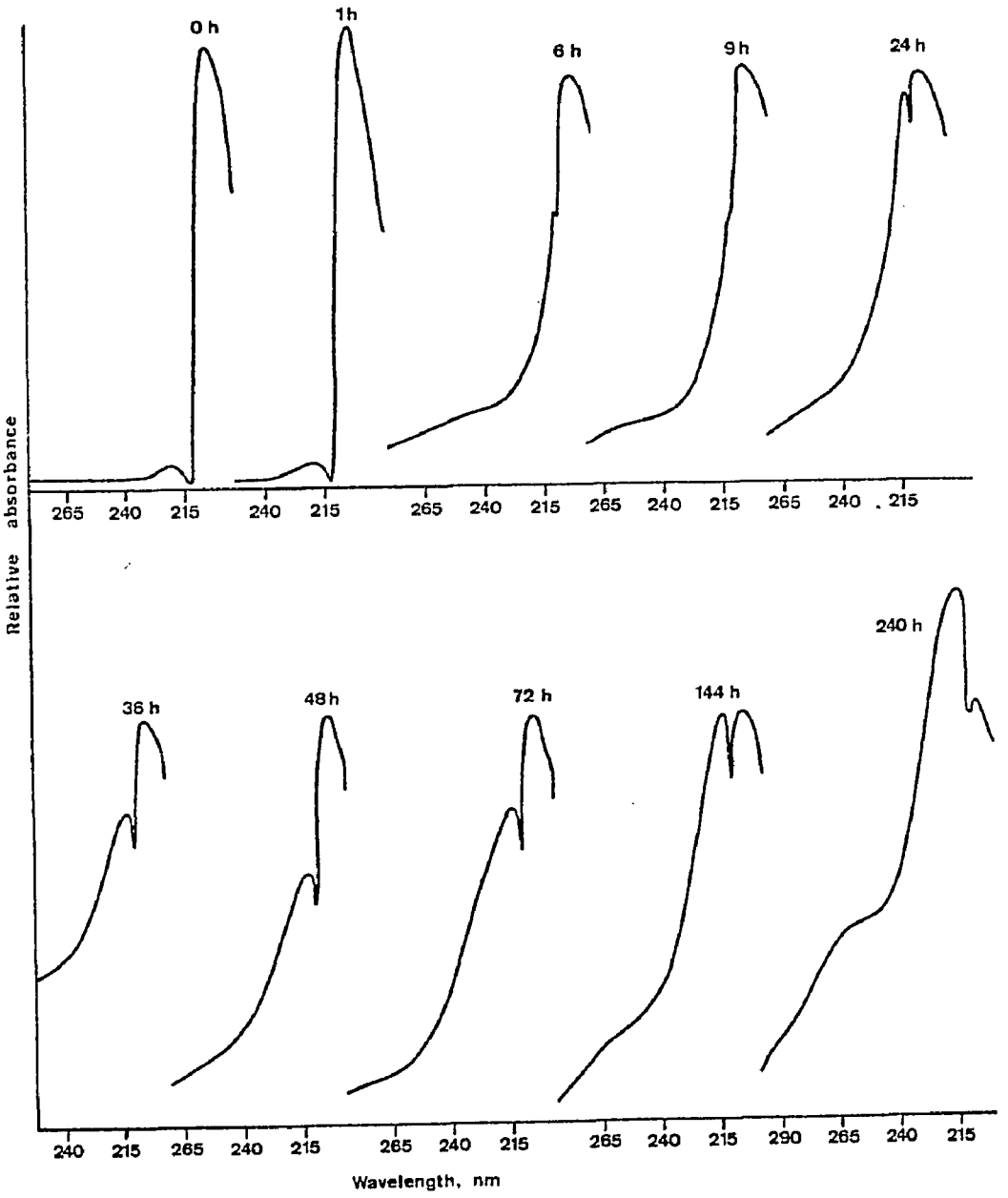

0 h
1 h
6 h
9 h
24 h
36 h
48 h
72 h
144 h
Relative absorbance
240 215 240 215 265 240 215 265 240 215 265 240 215
240 215 265 240 215 290 265 240 215 290 265 240 215
Wavelength, nm

Fig. 49. Ultraviolet spectroscopy of UB-M9 medium, 1 X 10^{-4}M Ni inoculated with A. marinus. Samples of medium were taken, filtered to remove light scattering material and UV scanned from 340 to 190 nm, blanked against uninoculated medium.


0 h
1h
6 h
9 h
24 h
36 h
48 h
72 h
144 h
240 h
Relative absorbance
265 240 215
240 215
265 240 215
265 240 215
265 240 215
265 240 215
240 215
265 240 215
265 240 215
265 240 215
290 265 240 215
Wavelength, nm

medium. It was considered likely that the peak was due to the acidic glucose metabolite produced by A. marinus. Gluconic acid, which was considered as a candidate for the acidic metabolite, gave a UV peak qualitatively similar to A. marinus in UB-M9 but only at concentrations which were too high. There were definite changes in the UV spectrum of UB-M9 medium during the growth of A. marinus but none could definitely be associated with the presence or absence of Ni. Medium conditioning was not due to cell leakage of nucleic acids and proteins as the absorbance peaks characteristic of these components did not appear until mid to late log growth phase.

Partitioning of Ni During Growth

Although previous experiments with 2216E LN medium had demonstrated that Ni uptake by A. marinus, if any, was below the limits of detection, it was undertaken to examine whether during the growth of A. marinus in UB-M9 medium there was any change in the physical form of Ni whether by precipitation or cell uptake. Changes in the chemical form of Ni in solution also were monitored by determining the extent to which Ni penetrated a dialysis membrane. Ni which was bound to organic molecules of molecular weight greater than the porosity of the dialysis membrane used would be unable to penetrate through the membrane and be retained.

Using the ^{63}Ni radioisotope it was undertaken to study the physical form of Ni in the medium during growth. Flasks of 2×10^{-5}M Ni labeled with 599 DPM/ml were inoculated with A. marinus and the growth monitored. The growth phase of the

experiment was performed in conjunction with the UV absorbance procedure and the growth curve of that experiment at 2 X 10^{-5}M Ni (Fig. 46) should be referred to for this experiment. At intervals during the lag and growth of the organism a flask was removed from the shaker and its entire volume filtered through a 0.22-μm Millipore membrane filter which was washed once with 75 % KASW. The ^{63}Ni activity of the filter and of duplicate 1-ml aliquots of the filtrate was determined via LSC. The activity per ml of solution of particulate material was calculated. Changes in the chemical speciation of the soluble Ni were examined by dialyzing 10-ml aliquots of the filtrate against an equal volume of 75 % KASW through Spectrapore 3 dialysis tubing (nominal pore size 3500 daltons). Dialysis was performed at 4°C and carried to equilibrium (approximately 24 h). Following dialysis the radio-Ni activity of the dialyzate and retentate were determined (Table 36).

The times from 0 to 28 h represented the lag phase, 72 h was mid-log, and 144 h and 240 h were stationary phase. At each time the material retained on the filter was less than 1 % of the solution activity per ml. The material retained by the filter represented either Ni taken up by the cells or precipitated Ni. There was little loss of Ni from solution by uptake or precipitation. There was considerable variation in the activity per ml of the filtrate but most values fell within one standard deviation of the mean. The ratio of retentate to dialysate activity is a measure of the effective molecular weight of the Ni. If the Ni was in a form which could readily permeate the dialysis tubing such as free Ni

Table 36. Dialyzability of ^{63}Ni in UB-M9 medium with 2 X 10^{-5}M Ni during growth of A. marinus

			^{63}Ni counts DPM/ml dialysis[c]		
Time, h	Particulate[a]/ml	solution/ml	retained	diffused	R:D[d]
0^-	1.37 ± 0.12	634 ± 54.7	351 ± 9.2	336 ± 36	1.044
0^+	1.55 ± 0.54	605 ± 12.9	370 ± 24.7	396 ± 2.8	0.935
6	4.74 ± 3.85	580 ± 13.4	308 ± 2.1	332 ± 22	0.927
9	4.02 ± 3.84	657 ± 87.6	428 ± 204	469 ± 220	0.923
24	1.64 ± 0.03	629 ± 49.7	345 ± 7.1	324 ± 31.1	1.064
36	1.39 ± 0.49	538 ± 42.7	387 ± 23.3	687 ± 24.7	0.563
48	2.19 ± 0.58	808 ± 230	356 ± 14.1	582 ± 210	0.611
72	4.45 ± 1.27	598 ± 201	315 ± 4.24	485 ± 226	0.687
144	2.59 ± 2.67	771 ± 187	548 ± 187	486 ± 157	1.127
240	0.31 ± 0.16	699 ± 169	350 ± 35.3	515 - 213	0.679

0^- = uninoculated

0^+ = inoculated, 1.0 ml EQ

[a] retained on filter.

[b] ± 1 standard deviation

[c] through 3500 mw dialysis tubing

[d] ratio of counts retained to counts diffused

ions or Ni bound by organic compounds of molecular weight less than 3500 daltons, the ratio of retained to dialyzed Ni activities would be 1. If the Ni were bound by organic compounds of molecular weight greater than 3500 such as by a small protein, it would be retained by the dialysis membrane and the R:D ratio would be greater than one. At all times up to 36 h the ratio was essentially 1. At 144 h the ratio was slightly greater than 1 at the other times it was less than 1. As there is no condition under which dialysis of Ni should proceed to a point that more had diffused out than was present within the membrane, those values were erroneous perhaps due to machine induced error in the LSC counting. This experiment produced no evidence for the uptake of Ni by the organisms, the precipitation of Ni by the organisms, or the binding of Ni by organic molecules of molecular weight greater than 3500 daltons.

Uptake of Ni

The above experiment and the previously mentioned 2216E LN Ni uptake experiments indicated very low levels of metal uptake by cells of A. marinus. A final attempt to measure the extent of the uptake, if any, of Ni (as ^{63}Ni) by the organism was made in order to establish whether Ni uptake into cells had a role in the conditioning of the medium. Previous investigators studying A. marinus uptake of Ni (Cobet, 1968) and Zn (Jones, Royle, and Murray, 1976) had indicated significant cellular contents of these metals. Uptake increased at or

near the metal concentration giving growth inhibition in complex medium.

A final attempt was made to assay the extent of Ni uptake by A. marinus. In addition to the use of UB-M9 medium rather than 2216E LN, these ^{63}Ni uptake experiments differed from those used previously in that both the density of cells and the activity of ^{63}Ni were much higher. To 25 ml volumes of broth medium were added Ni concentrations ranging from 0 to 1 X 10^{-4}M, and labeled with from 0.01 to 0.2 μCi/ml of ^{63}Ni dependent on Ni concentration. Cells of A. marinus were harvested by filtration from a 24 h culture and resuspended to an equivalent OD of 31.6 (3286 μg/ml dry cell mass). 3183 μg of cells were added to each flask. At 15, 60, 120, 180, 540, and 1200 min incubation (25°C static) the Ni uptake was measured by filtering a 2 ml aliquot through a 25 mm diameter 0.4-μm porosity Nuclepore filter, washing 2X with 5 ml 75 % KASW and counting the filter via LSC. At intervals of 120, 540, and 1200 min the OD of each flask was measured to compensate for growth. Uptake was calculated as μg Ni/μg cells. A second experiment was run as above using 1093 μg cells/flask. At a Ni concentration of 1 X 10^{-6}M, three additional treatments were added to assay any energy dependence of the uptake process: cells inhibited by heat (100°C, 1 min), 1 X 10^{-2}M NaN_3, and 1 X 10^{-3}M NaCN.

The data from the two replicate sets of experiments are presented in Table 37 as the mean ± 1 standard deviation. Where a standard deviation is absent, that time and/or treatment was used in only one of the two experiments. The data is

Table 37. Uptake of Ni by *A. marinus* in UB-M9 medium.

Time, h[a]	0 M[b] (2.73×10^{-3})[b]	1×10^{-7} (8.60×10^{-3})	1×10^{-6} (6.14×10^{-2})	1×10^{-5} (5.87×10^{-1})	1×10^{-4} (5.87)
1/4	$4.92 \pm 4.40 \times 10^{-7}$[c]	$3.45 \pm 7.84 \times 10^{-7}$	$4.83 \pm 3.29 \times 10^{-6}$	$1.95 \pm 1.14 \times 10^{-5}$	$9.13 \pm 5.19 \times 10^{-5}$
	6.41 ± 0.46[d]	6.11 ± 0.42	5.37 ± 0.32	4.75 ± 0.27	4.17 ± 0.40
	180 ± 161[e]	110 ± 90.9	78.9 ± 55.8	26.9 ± 28.4	15.5 ± 8.77
	14.1 ± 12.6[f]	8.58 ± 7.09	6.19 ± 4.22	2.10 ± 2.23	1.21 ± 0.69
1	$8.37 \pm 5.55 \times 10^{-7}$	$1.23 \pm 0.85 \times 10^{-6}$	$5.33 \pm 2.96 \times 10^{-6}$	$2.77 \pm 1.71 \times 10^{-5}$	$1.37 \pm 0.74 \times 10^{-4}$
	6.13 ± 0.31	5.96 ± 0.33	5.31 ± 0.25	4.16 ± 0.70	3.89 ± 0.25
	306 ± 203	144 ± 99	86.9 ± 48.2	37.9 ± 41.0	23.3 ± 12.5
	24.0 ± 15.9	11.3 ± 7.7	6.81 ± 3.78	2.97 ± 3.22	1.83 ± 0.98
2	4.56×10^{-7}	8.80×10^{-7}	3.95×10^{-6}	1.77×10^{-5}	9.10×10^{-5}
	6.34	6.05	5.40	4.75	4.04
	167	102	64.3	10.4	15.5
	13.1	8.00	5.04	0.816	1.22
3	$1.69 \pm 1.51 \times 10^{-6}$	2.09×10^{-6}	$9.43 \pm 6.60 \times 10^{-6}$	1.77×10^{-5}	9.10×10^{-5}
	5.88 ± 0.46	5.75 ± 0.37	5.08 ± 0.33	4.55 ± 0.19	3.88 ± 0.31
	618 ± 554	243 ± 184	154 ± 108	38.7 ± 37.7	25.3 ± 16.5
	48.5 ± 43.4	19.1 ± 14.4	12.0 ± 8.43	3.04 ± 2.96	1.83 ± 1.07

Table 37 Continued.

1×10^{-6}
(6.14×10^{-2})

Time, h	Control	Boiled	Azide	Cyanide
1	$5.33 \pm 2.98 \times 10^{-6}$	7.09×10^{-5}	7.43×10^{-6}	4.31×10^{-5}
	5.31 ± 0.25	4.15	5.13	436
	86.9 ± 48.2	1153	121	673
	6.81 ± 3.78	9.02	9.49	52.8
2	3.95×10^{-6}	-	-	-
	5.40	-	-	-
	64.3	-	-	-
	5.04	-	-	-
3	$9.43 \pm 6.60 \times 10^{-6}$	5.07×10^{-5}	1.08×10^{-5}	4.26×10^{-5}
	5.08 ± 0.33	4.29	4.97	4.37
	154 ± 108	826	176	694
	12.0 ± 8.43	64.8	13.8	54.4
8	$1.15 \pm 0.06 \times 10^{-5}$	4.5×10^{-5}	9.63×10^{-6}	2.93×10^{-5}
	4.93 ± 0.02	4.38	5.02	4.55
	187 ± 9	676	157	461
	14.7 ± 0.7	53.0	12.7	36.2

Table 37 Continued.

Time, h	0 M (2.73×10^{-3})	1×10^{-7} (8.60×10^{-3})	1×10^{-6} (6.14×10^{-2})	1×10^{-5} (5.87×10^{-1})	1×10^{-4} (5.87)
8	$1.40 \pm 0.23 \times 10^{-6}$	$2.85 \pm 3.18 \times 10^{-6}$	$1.15 \pm 0.06 \times 10^{-5}$	$5.38 \pm 1.09 \times 10^{-5}$	$3.75 \pm 0.94 \times 10^{-4}$
	5.86 ± 0.07	5.54 ± 0.05	4.93 ± 0.02	4.27 ± 0.09	$3.48 \pm 0.13 \times 10^{-4}$
	512 ± 83	332 ± 37	187 ± 9	57.4 ± 29.9	57.4 ± 16.8
	40.2 ± 6.5	26.0 ± 2.9	14.7 ± 0.7	4.5 ± 2.4	4.50 ± 1.31
24	$1.24 \pm 0.99 \times 10^{-6}$	$1.56 \pm 0.54 \times 10^{-6}$	$6.30 \pm 0.23 \times 10^{-6}$	$1.95 \pm 0.71 \times 10^{-5}$	$1.30 \pm 0.62 \times 10^{-4}$
	5.98 ± 0.39	5.76 ± 0.23	5.20 ± 0.01	4.72 ± 0.16	3.91 ± 0.21
	455 ± 361	214 ± 109	102.5 ± 3.5	19.6 ± 7.2	22.1 ± 10.5
	35.7 ± 28.3	16.7 ± 8.6	8.03 ± 0.28	1.54 ± 0.57	1.73 ± 0.83

	1×10^{-6} (6.14×10^{-2})			
Time, h	Control	Boiled	Azide	Cyanide
1/4	$4.83 \pm 3.29 \times 10^{-6}$	8.26×10^{-5}	8.57×10^{-6}	7.16×10^{-6}
	5.37 ± 0.32	4.08	5.07	5.14
	78.9 ± 55.8	1345	140	117
	6.19 ± 4.22	105	11.0	9.18

Table 37 Continued.

		1×10^{-6} (6.14×10^{-2})		
Time, h	Control	Boiled	Azide	Cyanide
24	$6.30 \pm 0.23 \times 10^{-6}$	2.67×10^{-5}	2.04×10^{-5}	1.56×10^{-5}
	5.20 ± 0.01	4.57	4.69	4.81
	102.5 ± 3.5	435	332	254
	8.03 ± 0.28	34.1	26.0	19.9

[a]at time cells filtered and ^{63}Ni taken up counted by LSC.

[b]top Ni value = molarity added; bottom value = calculated value (µg/liter) from added value + Ni present in 0 Ni added media.

[c]µg Ni/µg dry cells; cell values ± are standard deviation.

[d]-log µg Ni/µg cells.

[e]concentration factors µg Ni/µg cells ÷ µg Ni/µg medium.

[f]concentration factor corrected for cell water content.

presented as four values. The first, μg Ni/μg cells, is the concentration of the metal as a fraction of the cell dry weight. This was calculated from the measured activity of the cells on the filter, the known ratio of DPM to μg Ni in the medium, the volume of medium filtered, the OD of the medium, and the ratio of OD to dry cell mass (see Measurement of Growth). The cell concentration of Ni tended to increase both with the Ni concentration of the medium (though not proportionately) and with the incubation time. At the highest concentration (1×10^{-4}M Ni, 8 h), the cells were slightly less than 0.04 % Ni by weight (by contrast, at that Ni concentration the medium solids were 0.018 % Ni). The second value is the log of the Ni content. Because of the wide dynamic range of the Ni content values, the log transformation was used to plot the uptake versus time (Figs. 50 and 51). The third value is the concentration factor. It was calculated by dividing the cell content of Ni by the medium content (converted to identical units of μg Ni/μg medium). The concentration factor usually is reported as such in the literature using the metal content of the organism on a dry cell mass basis. For comparison, the fourth value is the concentration factor corrected to a wet cell mass basis by the ratio of wet to dry cell mass which was derived in Measurement of Growth.

Significant and quantifiable uptake was found but the magnitude of the uptake was very low. Calculated on the basis of cell bound Ni as % of total medium Ni (data not shown), the uptake was from a maximum of 0.4 to 0.02 % with the greatest removal of Ni from solution being at 0 Ni added. Unlike the

Fig. 50. Ni uptake by A. marinus in UB-M9 medium at 25°C, incubation time (h) versus -log Ni concentration.

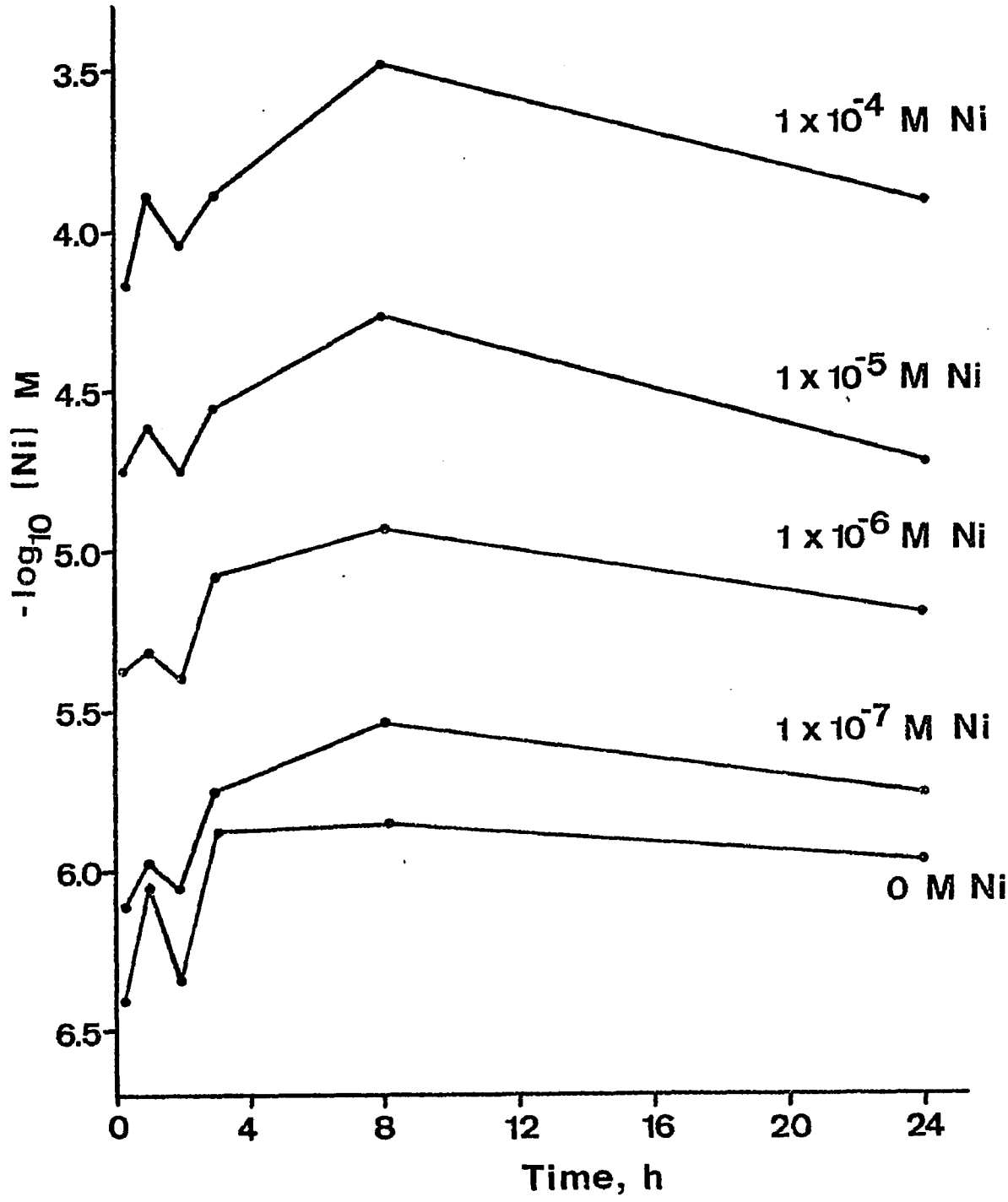
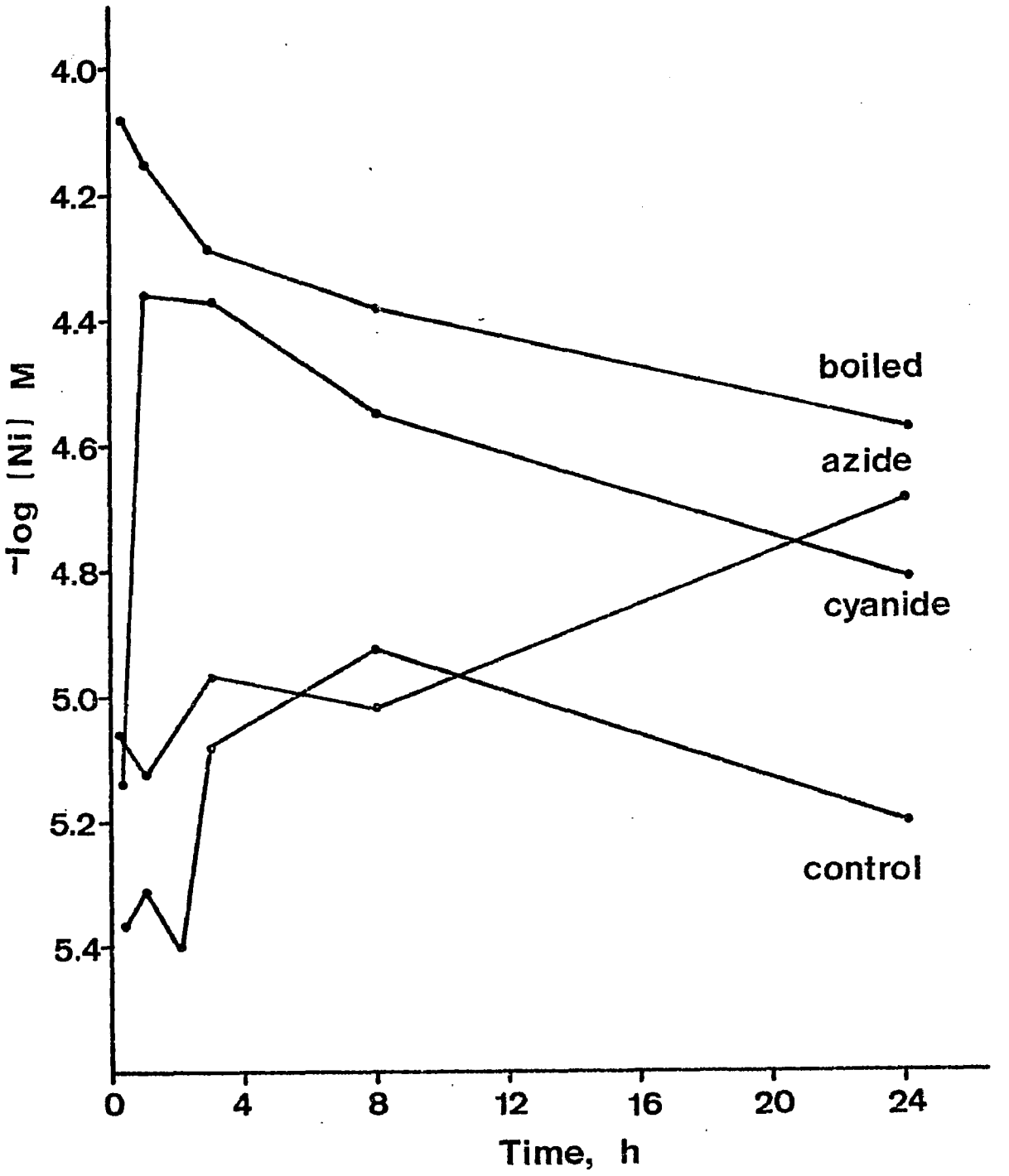

3.5
4.0
4.5
5.0
5.5
6.0
6.5
$-\log_{10}$ [Ni] M
1×10^{-4} M Ni
1×10^{-5} M Ni
1×10^{-6} M Ni
1×10^{-7} M Ni
0 M Ni
0
4
8
12
16
20
24
Time, h

Fig. 51. Effect of metabolic inhibitors on Ni uptake by A. marinus in UB-M9 at 25°C, 1 X 10^{-6}M Ni.


4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
-log [Ni] M
boiled
azide
cyanide
control
0
4
8
12
16
20
24
Time, h

previously reported metal uptake studies with A. marinus, relative uptake decreased with increasing metal concentration. The corrected concentration factor fell to near 1 at the highest Ni concentration. A concentration factor of 1 would indicate no uptake.

The plot of the log of cellular Ni concentration versus incubation time (Fig. 50) showed that the uptake of Ni was a slow process reaching saturation only after about 8 h. In all cases the Ni concentration subsequently dropped somewhat. The difference in cell Ni content between the first incubation time studied (15 min) and the Ni uptake peak (8 h) was about 0.5 log units or about 3 fold. The pattern of uptake with time in the case of the three inhibitory treatments was rather different (Fig. 51). The cells killed by boiling had reached or passed their uptake peak by 15 min incubation and their Ni content fell thereafter. Azide-inhibited cells showed a roughly linear increase in Ni content during incubation, while cyanide inhibited cells showed a very rapid rise between 15 and 60 min incubation followed by a dropoff during further incubation. These results with the inhibitors were interpreted as indicating that an intact and metabolically active cell was capable of limiting the uptake of Ni. Boiled cells had high concentration factors and cellular Ni concentrations perhaps due to disruption of the cell permeability barrier which allowed Ni to be adsorbed at cell sites not exposed in the intact cell. Metabolic inhibition of the cells by cyanide and azide showed no energy dependence of uptake. Indeed, there may have been an energy dependent Ni exclusion mechanism. This would explain

the higher Ni concentrations reached in the inhibited cells compared to the control cells.

Ultrafiltration

The dialysis experiment used a membrane of c. 3500 dalton porosity. While such a membrane is totally retentive for proteins and other macromolecules, it is non-retentive for the very significant group of molecules which include sugars, amino acids, nucleotides, organic acids, and lower polymers of them. The range of dialysis as a means of molecular separation and concentration can be extended downward to include many of the above compounds by using the technique of ultrafiltration which uses a very thin dialysis membrane supported by a porous substrate, and employs pressure to push the solvent (water) and solutes smaller than the membrane cut-off through into the permeate even against a osmotic pressure barrier. Ultrafiltration membranes are available having porosities as low as 500 daltons (Amicon UM-05).

Initial experiments designed to test filter performance and the sealing ability of the membrane filter holder (Nuclepore 90-mm radial flow cell) were performed using bovine serum albumin fraction V (Calbiochem) as a high molecular weight tracer. There was essentially no permeation of the protein through the membrane and 70 % or greater retention of the solute. That the retention was calculated as less than 100 % may have indicated that there was adsorption of the protein onto the membrane.

Having thus demonstrated the proper performance of the filter, repeated attempts were made to demonstrate the presence of Ni-organic complexes of sufficiently large size to be retained by the membrane. In both uninoculated and lag and growth phase culture filtrates of UB-M9 medium with A. marinus, no evidence (in the form of a higher concentration of Ni, as ^{63}Ni tracer, in the filter retentate during the course of filtration) for Ni-organic complexes was found. Further, it was consistently found that there was a deficit of Ni in the permeate solution which could not be explained by either membrane adsorption of Ni or by dilution into the sub-filter dead volume of the apparatus. Ultrafiltration was, therefore, abandoned.

Notwithstanding the technical difficulties with the technique, there was at no point any evidence for the occurrence of Ni-organic complexes of molecular weight greater than 500 daltons. This would imply that either the organic species complexing the Ni were less than 500 dalton molecular weight or that they were sufficiently labile that they underwent dissociation at the membrane surface allowing the freed Ni to pass through the membrane while the organic moiety remained in the retentate. The possibility that the evidence might be interpreted as proof for the absence of Ni complexation was not overlooked.

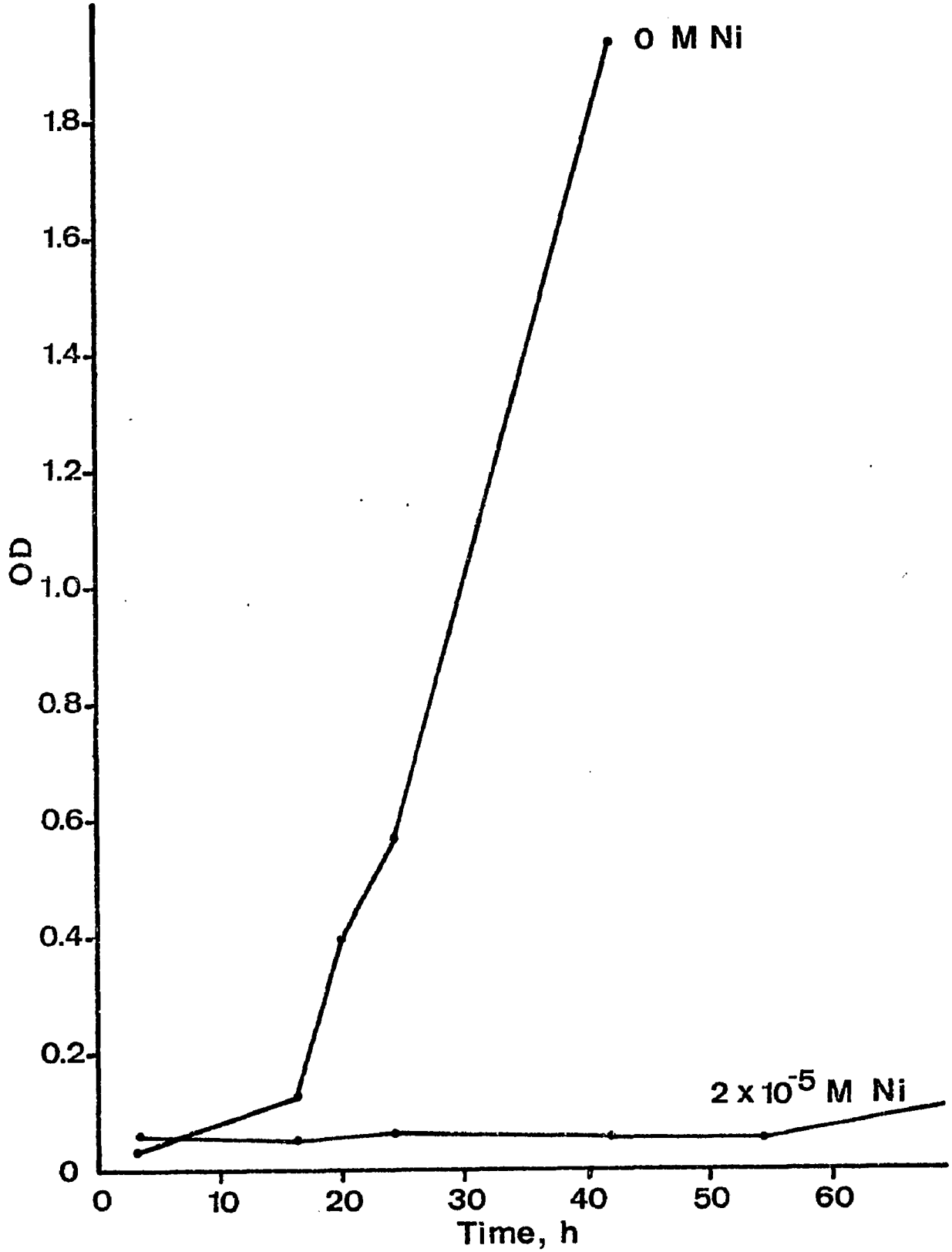
Gel Filtration Chromatography

As was the case above when ultrafiltration was used to detect metal complexation, one of the methods by which

organometallic complexation may be detected is by the fact that such complexation will raise the effective molecular weight of the complexed metal from that of the metal itself to that of the metal plus the organic moiety. Thus a 600 dalton chelating compound which had complexed a single ion of Ni would have an effective molecular weight of 660 and might be readily distinguished from the free metal by an appropriate technique. Gel filtration chromatography is a technique which separates organic compounds on the basis of their molecular weight such that molecules larger than a specified resin exclusion limit elute first from the column (in the "void volume") and smaller molecules separate inversely to their molecular weight. The resin used was Bio-gel P-2 which has an exclusion limit of 1800 daltons. Free Ni was expected to be retarded strongly by the resin. Ni complexed by a small organic compound would elute at a lower volume while Ni complexed by a molecule of 1800 dalton or greater molecular weight would elute first from the column. Thus, complexation of Ni would lead to the shifting of the peak of Ni activity towards lower elution volumes and/or the appearance of one or more peaks below the "free" Ni peak.

An experiment was prepared using UB-M9 medium with Ni concentrations of 0 and 2 X 10^{-5}M Ni and labeled with 35,000 DPM/ml of ^{63}Ni. The flasks were inoculated with 1.0 ml EQ of an 18 h washed A. marinus starter culture and the growth monitored (Fig. 52). At intervals one of the flasks which had been inoculated simultaneously was removed from the shaker, filtered through a 0.4-μm Nuclepore membrane, and the sterile

Fig. 52. Growth, as OD, of A. marinus in UB-M9 medium with 2×10^{-5}M Ni for gel permeation chromatography.


0 M Ni
2 x 10-5 M Ni
OD
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Time, h

filtrate stored at 4°C until chromatographed. The chromatography results for 0 Ni are shown in Fig. 53, 2 X 10^{-5}M Ni in Fig. 54. In the case of 0 Ni there was a slight down shifting of the Ni peak after 24 h, at which point the culture was in mid-log growth. There was no detectable peak shifting during lag. The same was true of the data for 2 X 10^{-5}M Ni.

From these results two possible conclusions were drawn. Either there were no organic complexes of Ni formed which fell into the operational range of the resin, or the complexes were sufficiently labile that they underwent dissociation during chromatography and the Ni eluted separately from its originally complexing organic molecule. Information provided by the resin manufacturer indicated that tailing of the peaks, as is evident in both figures, is characteristic of interaction between the resin and the solute as by adsorption. It was possible that the resin, acting as a cation exchanger, was out-competing the metal complexing agent produced by the organism.

HMBO Detection

The heavy metal binding organic (HMBO) compound detection system using chromatography on the chelating ion exchange resin Chelex-100 was used to detect the presence of high stability organometallic complexes in the culture filtrate. During chromatography past the chelating resin, free Ni, or Ni bound by organic compounds of stability constants appreciably lower than the resin functional groups (aminodiacetate, stability constant comparable to EDTA), would be taken up by the resin. The only metal which would appear in the column

Fig. 53. Gel permeation chromatography of 0 Ni UB-M9 medium *A. marinus* culture supernatents labeled with ^{63}Ni and harvested at the times indicated. At intervals of 1/2 void volume/ml, aliquots of eluate were taken and the activity determined.

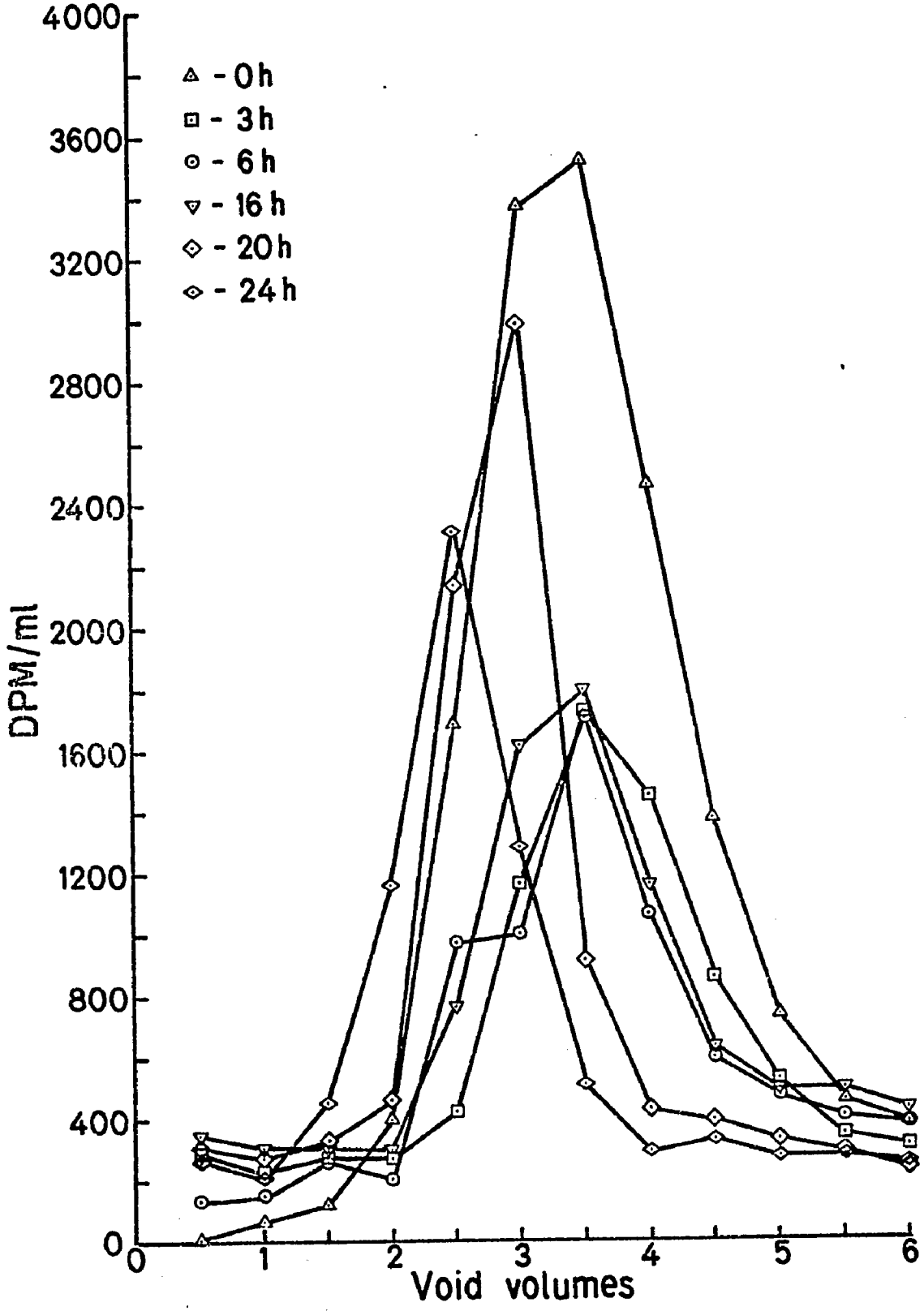
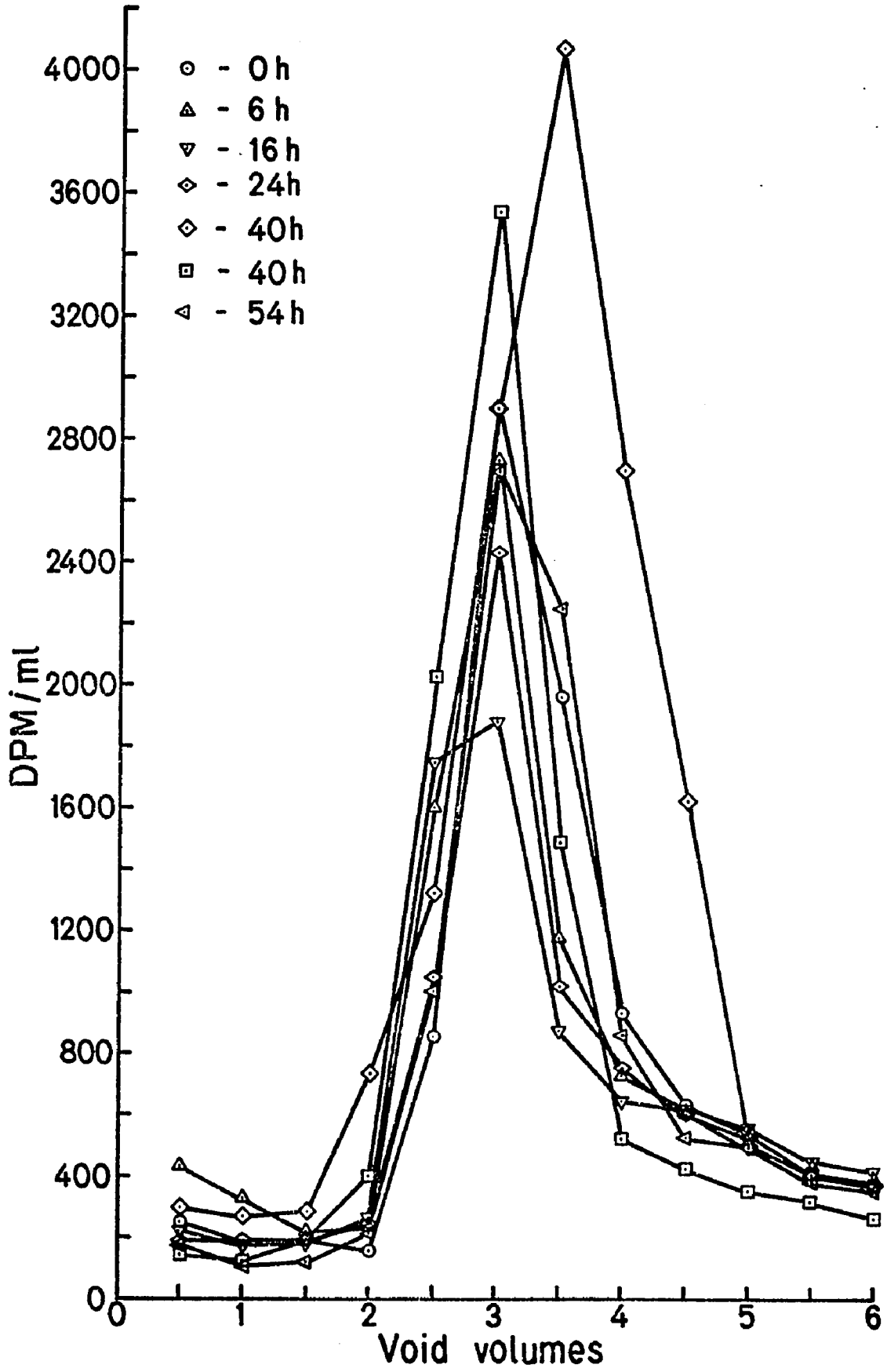

4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
DPM/ml
△ - 0h
□ - 3h
○ - 6h
▽ - 16h
◇ - 20h
◇ - 24h
0
1
2
3
4
5
6
Void volumes

Fig. 54. Gel permeation chromatography of 2 X 10^{-5}M Ni UB-M9 medium A. marinus culture supernatents labeled with ^{63}Ni and harvested at the times indicated. At intervals of 1/2 void volume/ml, aliquots of eluate were taken and the activity determined.


4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
DPM/ml
0
1
2
3
4
5
6
Void volumes
- 0h
- 6h
- 16h
- 24h
- 40h
- 40h
- 54h

effluent was that bound by complexing agents of stabilities equivalent to or greater than the resin.

The results of the initial experiment using Chelex-100 resin are presented in Fig. 55. Chelex-100 resin was unsaturated after 1 X 10^{-4} mole of Ni had been removed. The resin removed 99.3 % or more of the input activity (by comparison of input and effluent counts), and 105 % of the input activity was recovered upon elution of the column with 1 N HNO_3.

An initial experiment was run using the Chelex-100 resin system to detect the presence of the strong chelating agent EDTA. As was previously discussed, such a synthetic chelator may be a useful model of the medium conditioning agent. A sample of 75 % KASW was made to 5 X 10^{-5}M with Ni and 3.2 X 10^5 DPM/ml of ^{63}Ni, and aliquots were spiked with EDTA molarities of 0, 1 X 10^{-8}, 1 X 10^{-7}, 1 X 10^{-6}, and 1 X 10^{-5}M. The samples were then chromatographed on Chelex-100, the input and effluent activity determined by LSC, and the HMBO molarity calculated from the ratio of effluent to input counts multiplied by the Ni molarity. The results are presented in Table 38. The technique was capable of detecting better than 74 % of added EDTA and was 90 % or better at EDTA concentrations greater than 1 X 10^{-7}M.

Experiments run in 75 % KASW and QH_2O without added chelating agent which were designed to detect any background of HMBO gave counts twice or less of the background count of the counter and were considered to have HMBO content below detection.

Fig. 55. Resin exchange capacity of a 1.5 X 3 cm column of Chelex-100 resin. A 1 liter volume of ^{63}Ni labeled (8.2×10^{4} DPM) 1×10^{-4} M Ni QH_2O chromatographed on the column. At 100 ml volumes, samples were taken and the activity determined.

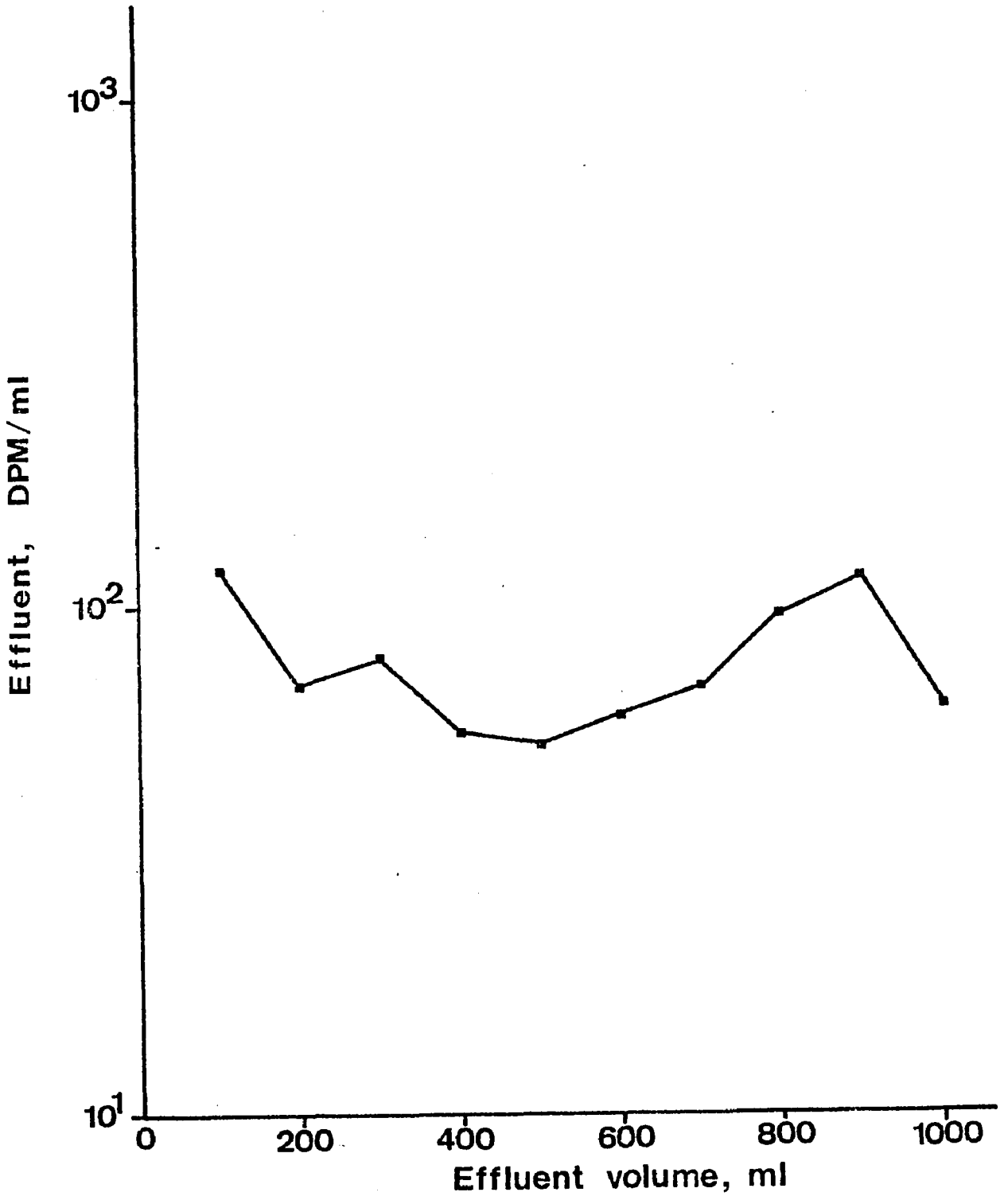

10^3
10^2
10^1
Effluent, DPM/ml
0
200
400
600
800
1000
Effluent volume, ml

Table 38. Quantitative detection of EDTA in 75 % ASW with 5×10^{-5}M Ni, Chelex-100 HMBO technique.

EDTA, M	Input[a]	Effluent[a]	HMBO M[b]	% Added Chelator
0	322900	24.4	3.78×10^{-9}	-
1×10^{-8}	322900	70.4	7.40×10^{-9}	74.0
1×10^{-7}	322900	593	8.85×10^{-8}	88.5
1×10^{-6}	322900	6426	9.86×10^{-7}	98.6
1×10^{-5}	322900	60659	9.36×10^{-6}	93.9

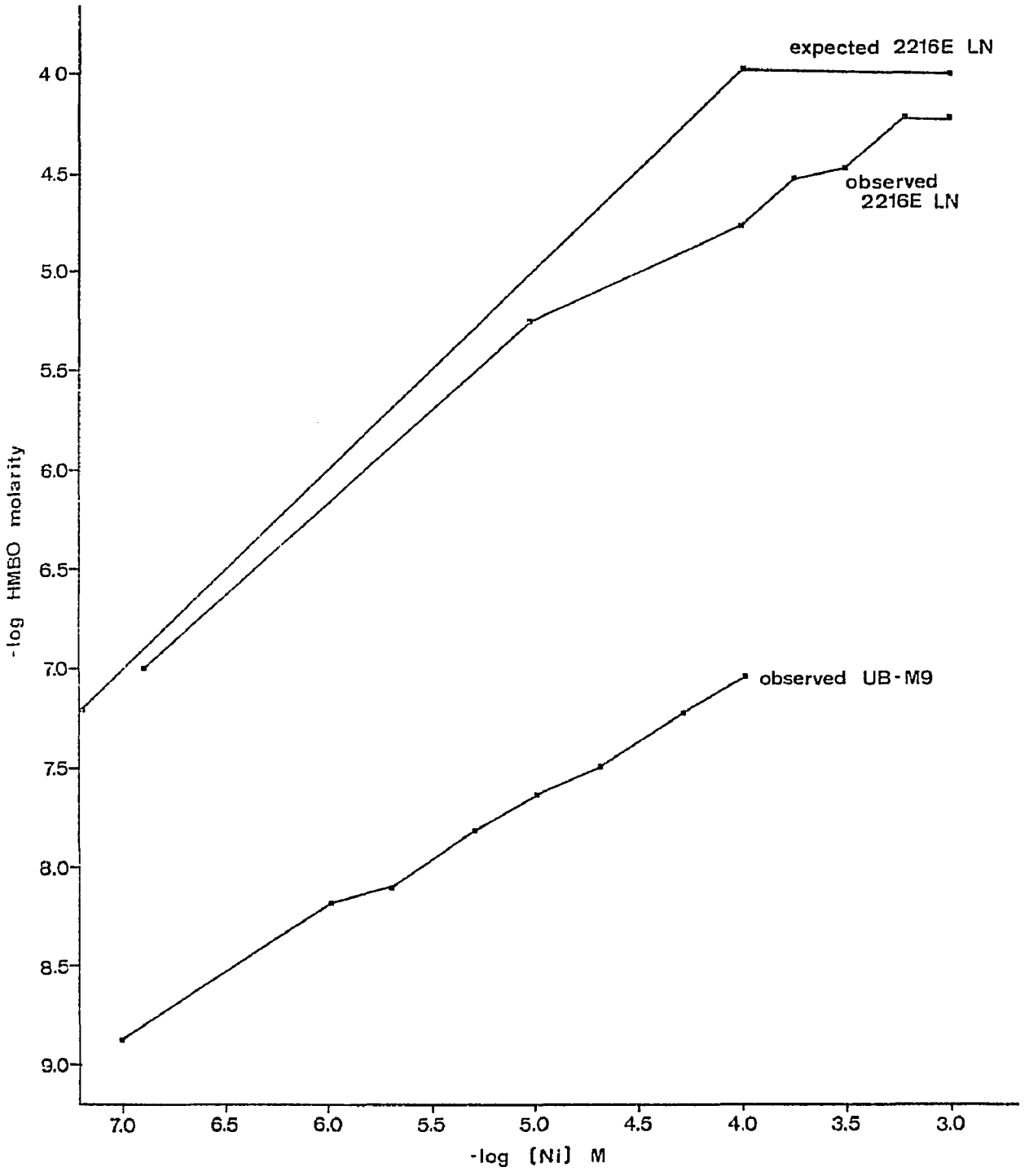
[a]activity measured in DPM/ml by LSC

[b]ratio of input to effluent $\times 5 \times 10^{-5}$M

The technique was applied to the detection of strong metal binding compounds in bacterial growth media. Samples of 2216E LN and UB-M9 media were prepared containing varying concentrations of Ni and labeled with ^{63}Ni. These were passed through the resin column and the activity in the effluent determined by LSC. The results for such an experiment using 2216E LN medium are shown in Fig. 56.

The expected line on the plot was calculated on the basis of the proposal that in this medium the point at which Ni toxicity appeared was that point at which the complexing capacity of the medium was exceeded and free Ni ion appeared. In that case all of the Ni added would be able to pass through the column up to the point at which the metal binding ligands of the peptone and yeast extract were saturated and the molarity of HMBO measured below the saturation point would be identical to the Ni concentration of the medium. At and above the saturation point only that molarity of Ni which was complexed by the organic ligands would pass through the column and the HMBO molarity measured would become constant. It would represent the actual metal binding capacity of the medium. The curve of observed HMBO molarity showed considerable deviation from the expected curve. Rather than a distinct break point, the curve showed a broad leveling. Further, even at very low Ni concentrations a considerable fraction of the Ni was in a form labile to the column. This deviation from the expected response was not due to the occurrence of ligand-resin exchange reactions during chromatography. A repetition of the same experiment using a higher rate of flow through the column which

Fig. 56. HMBO quantitation in 2216E and UB-M9 medium. Samples of ^{63}Ni labeled medium of Ni concentrations from 1 X 10^{-7} to 1 X 10^{-3}M Ni were chromatographed on Chelex-100. HMBO molarity was determined from the ratio of input to effluent activity X the Ni concentration. The expected curve showed the response if the media contained 1 X 10^{-4}M of a strong metal chelator (such as EDTA).


expected 2216E LN
observed 2216E LN
observed UB-M9
-log HMSO molarity
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
-log [Ni] M

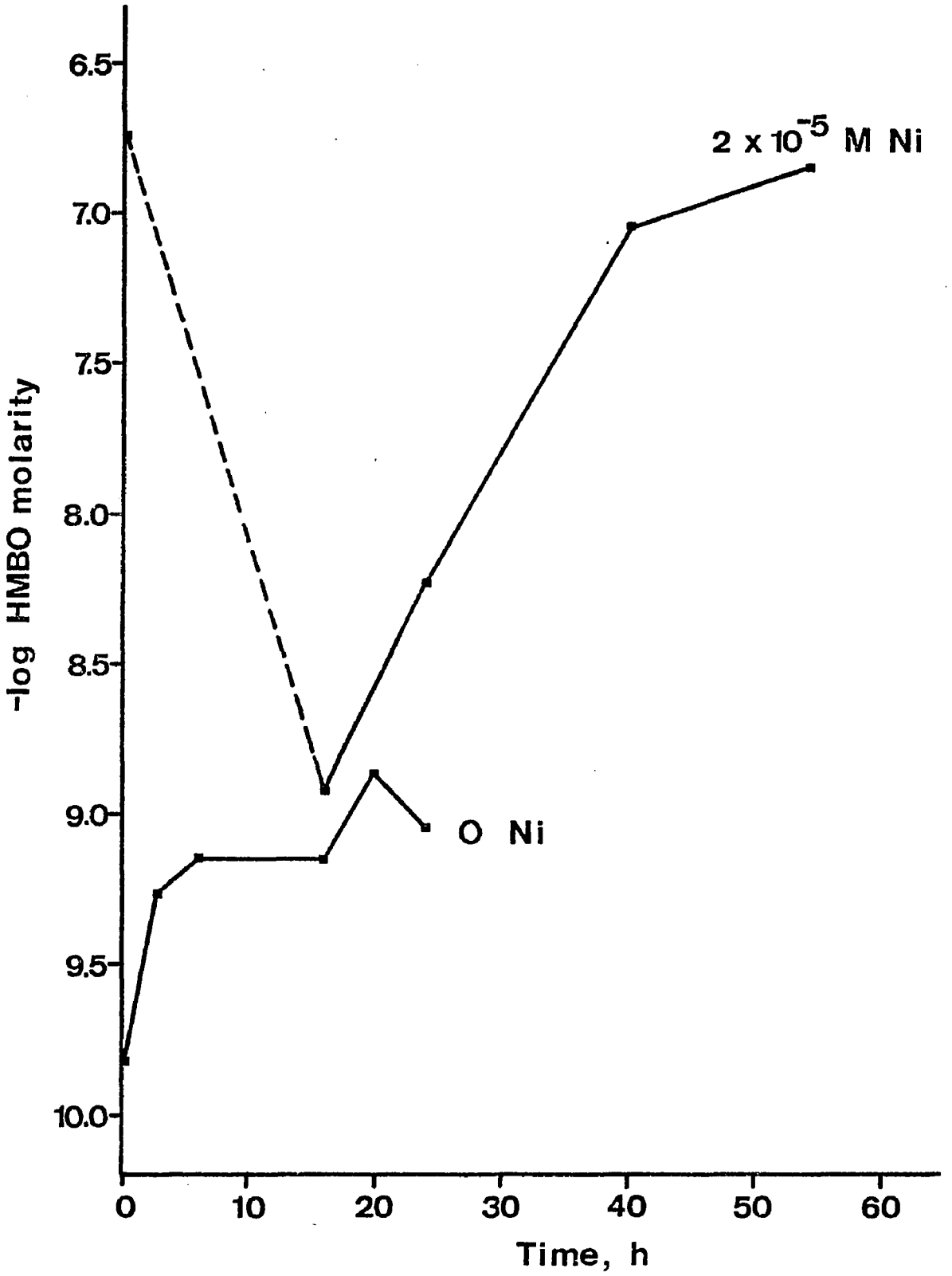
would reduce any exchange reactions gave essentially the same response.

Within the definition of technique, there was free Ni in 2216E LN medium even at Ni concentrations well below the toxicity point. The leveling of the observed curve at a HMBO molarity of -4.25 (5.62×10^{-5}M) might have indicated an effective strong metal binding capacity that was less than the point at which threshhold toxicity was observed. In this medium, mechanisms other than the presence or absence of strong Ni binding were regulating Ni toxicity.

A similar experiment, using UB-M9 medium produced the results shown in Fig. 56. As the Ni concentration rose there was a roughly linear rise in the measured HMBO molarity though the slope of the curve was less than 1. The HMBO concentration was consistently two or more orders of magnitude less than the Ni concentration. There was no evidence of any threshold leveling. It was impossible to define any HMBO concentration in this medium although there was evidence for some metal binding activity.

The HMBO technique was applied to the samples which were used for the gel filtration experiments (Fig. 57). These curves should be compared to the growth plots for this experiment in Fig. 52. Both 0 and 2×10^{-5}M Ni UB-M9 cultures of A. marinus showed measurable increase of HMBO molarity during the lag phase of growth. This increase, however, for neither culture approached the molarity of Ni present. The 0 Ni HMBO peak was only 2.8 % of the Ni molarity, and the 2×10^{-5}M Ni culture peaked at 0.69 % of Ni molarity. While

Fig. 57. Detection of HMBOs in UB-M9 medium with 0 and 2 X 10^{-5}M Ni during the growth of A. marinus. The 0 time (uninoculated) 2 X 10^{-5}M HMBO value was anomalously high and indicated by a dashed line.


6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
-log HMBO molarity
2 x 10⁻⁵ M Ni
O Ni
0
10
20
30
40
50
60
Time, h

this experiment gave good evidence for the production of strong metal binding agents during the lag of A. marinus in both the presence and absence of added Ni, the quantity of such compounds produced was far too low to account for the detoxification of Ni in UB-M9 medium during the organism's lag phase. However, this technique could detect quantitatively only very strong chelating agents (such as EDTA, $pK_m Ni=18.5$). Even such a moderately strong chelating agent as histidine ($pK_m Ni=8.7$) would not be detected. Thus, these results were negative only for very strong metal binding compounds, but did not answer the question of whether weaker complexing agents act in the conditioning of UB-M9 by A. marinus.

Differential Pulse Polarography

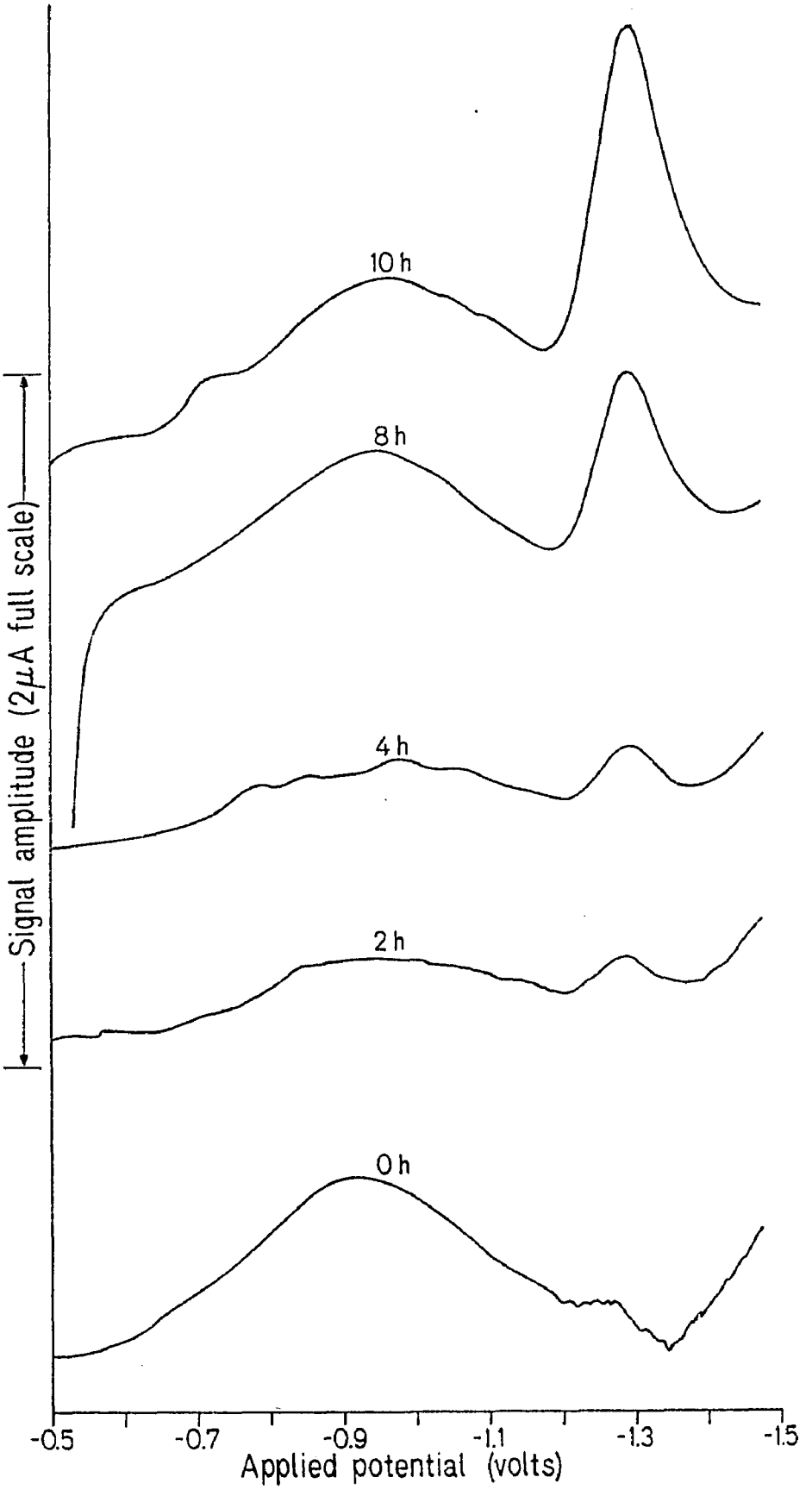
As has previously been discussed with relation to the polarography of known chelating agents, changes of the chemical speciation of a metal, particularly the formation of organo-metallic complexes, will modify the electrochemical behavior of the metal ion. A metal ion which gives a peak at a characteristic potential (c. -1.10 v for Ni) in its free or inorganically complexed form may have its characteristic potential changed by organic complexation. The peak will shift to a more negative potential reflecting the increased energy which must be exerted to free the ions from the organic moiety before it may be reduced at the electrode. Alternatively the peak size will be decreased. If the complex is very stable, or kinetically slow to dissociate, the metal ions cannot be freed to reduce at the electrode, and thus the bound

fraction of the total metal concentration will be "lost" from the metal peak. Therefore, the differential pulse polarographic technique was applied to the analysis of changes in the speciation of Ni in UB-M9 medium during the lag phase and growth of A. marinus.

While the polarographic technique used has the potential for great sensitivities (and for some metals the related anodic stripping technique is one of the most sensitive analytical techniques available) in the system used here with its relatively large added Ni concentrations, extreme sensitivities were not used. As was noted in the section on polarography of known chelating agents, concentrations of from 5×10^{-6} to 1×10^{-5}M Ni were readily detectable by pulse polarography and the Ni peak size was directly proportional to the Ni concentration added (Fig. 38; Table 34). The potential characteristic of Ni in UB-M9 medium without added complexing agents or modification by A. marinus growth was -1.09 v.

Initial experiments followed the growth of A. marinus in UB-M9 medium with added Ni. They showed the rapid development of a peak at -1.29 v during the lag which subsequently grew to extremely large size. It was suggested that the peak was an electroactive organic compound perhaps the metabolic by-product responsible for the pH drop during growth of the organism. The polarographic effect of growth of A. marinus on UB-M9 medium without added Ni was studied. The development of the peak can be seen in the polarograms shown in Fig. 58. The growth of the organism along with the pH and the size of

Fig. 58. Polarographic changes in UB-M9 medium during the growth of A. marinus. Scan range -0.5 to -1.5 v, 2 sec drop time, 1 mv/sec scan rate, 2 μA full scale sensitivity. Illustrated polarographs are of cultures at 0 to 10 h incubation (early to mid lag).


10 h
8 h
4 h
2 h
0 h
Signal amplitude (2μA full scale)
-0.5
-0.7
-0.9
-1.1
-1.3
-1.5
Applied potential (volts)

the peak is shown in Fig. 59. The polarographic peak had grown from essentially 0 to off-scale by the beginning of log phase growth and continued growing throughout log phase. This peak developed independently of Ni stress and was identified as an electroactive cell metabolite.

This metabolite peak was examined further to determine if it showed any polarographic interaction with Ni or other metals. Cu was used because it has a reduction potential of -0.15 v and was thus well separated from the metabolite peak as opposed to Ni, which was encroached on by the metabolite peak as it grew. Cu caused no changes in the metabolite peak when it was titrated (Fig. 60). Examination of the magnitude and reduction potentials showed the metabolite peak to occur at a stable -1.25 v potential and at a constant peak height while Cu varied from 0 added to 3.2×10^{-6}M. The Cu peak rose in direct proportion to the Cu added. By contrast, Zn, which has a reduction potential of -1.05 v, very near to the metabolite peak potential, interacted with the metabolite peak (Fig. 61). The Zn peak remained at a constant potential and grew proportionately with the added Zn. By contrast, the metabolite peak, while remaining approximately at its original size showed a consistent downward potential shift such that by 500 µg/liter (7.6×10^{-6}M Zn), the two peaks had merged into a single peak of -1.04 v. Peak interaction might arise either as a result of complexation or by the metal acting in a catalytic manner upon the organic functional group undergoing reduction at the observed potential. As Cu showed no interaction with the metabolite, the later hypothesis was more likely.

Fig. 59. Growth as OD, pH, and metabolite peak area (arbitrary units) of A. marinus grown in UB-M9 medium.

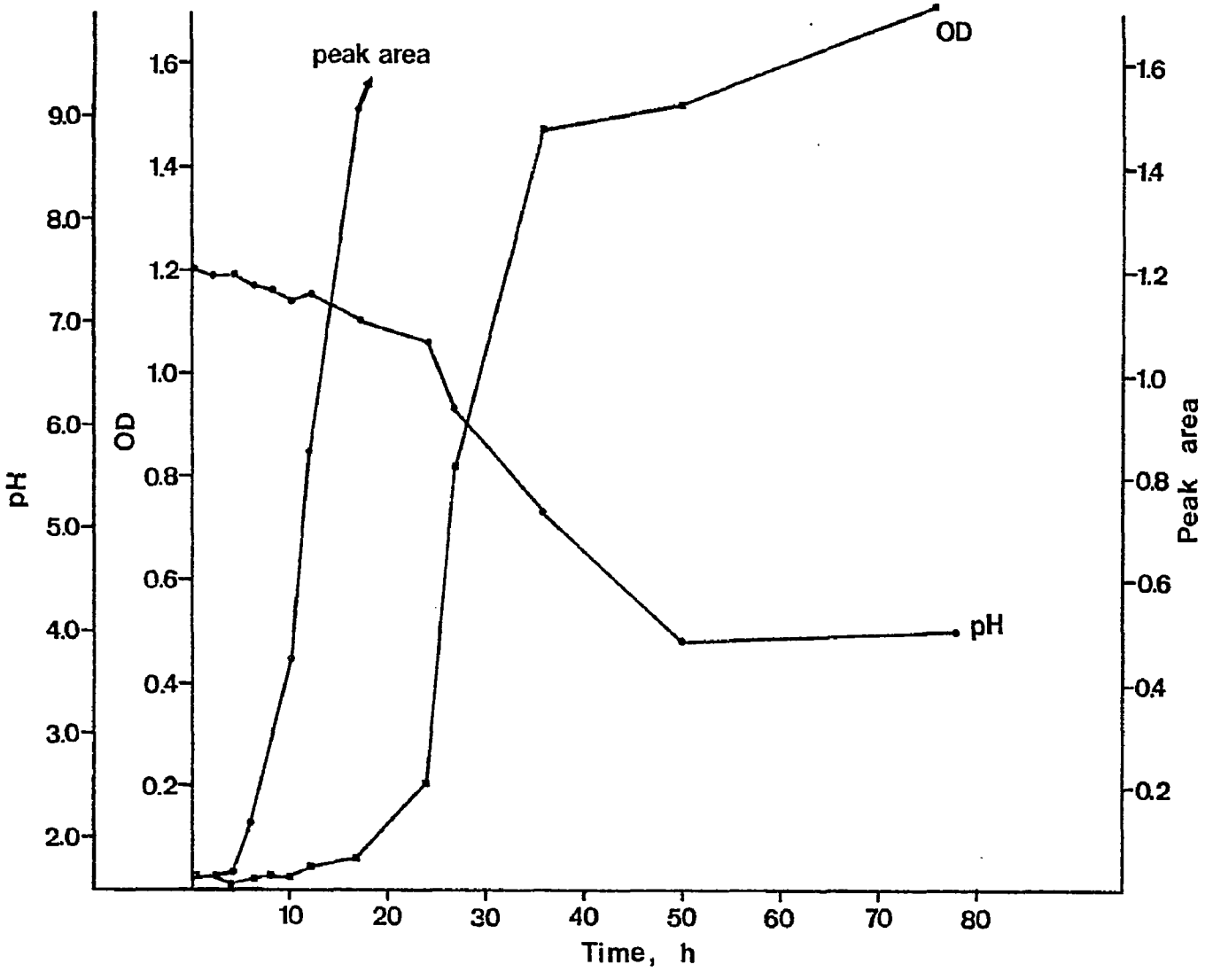

peak area
OD
pH
OD
Peak area
Time, h
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
10
20
30
40
50
60
70
80

Fig. 60. Polarographic effect of various Cu concentrations on the metabolite peak produced by A. marinus in UB-M9 (6 hr incubation): A) 0 added Cu, B) 6.3 X 10^{-7}M Cu, C) 1.3 X 10^{-6}M, D) 1.9 X 10^{-6}M, E) 3.2 X 10^{-6}M Cu. Polarographic parameters: 0.0 to -1.5 v, 2 sec drop time, 2 mv/sec scan rate, 1 μA full scale sensitivity.

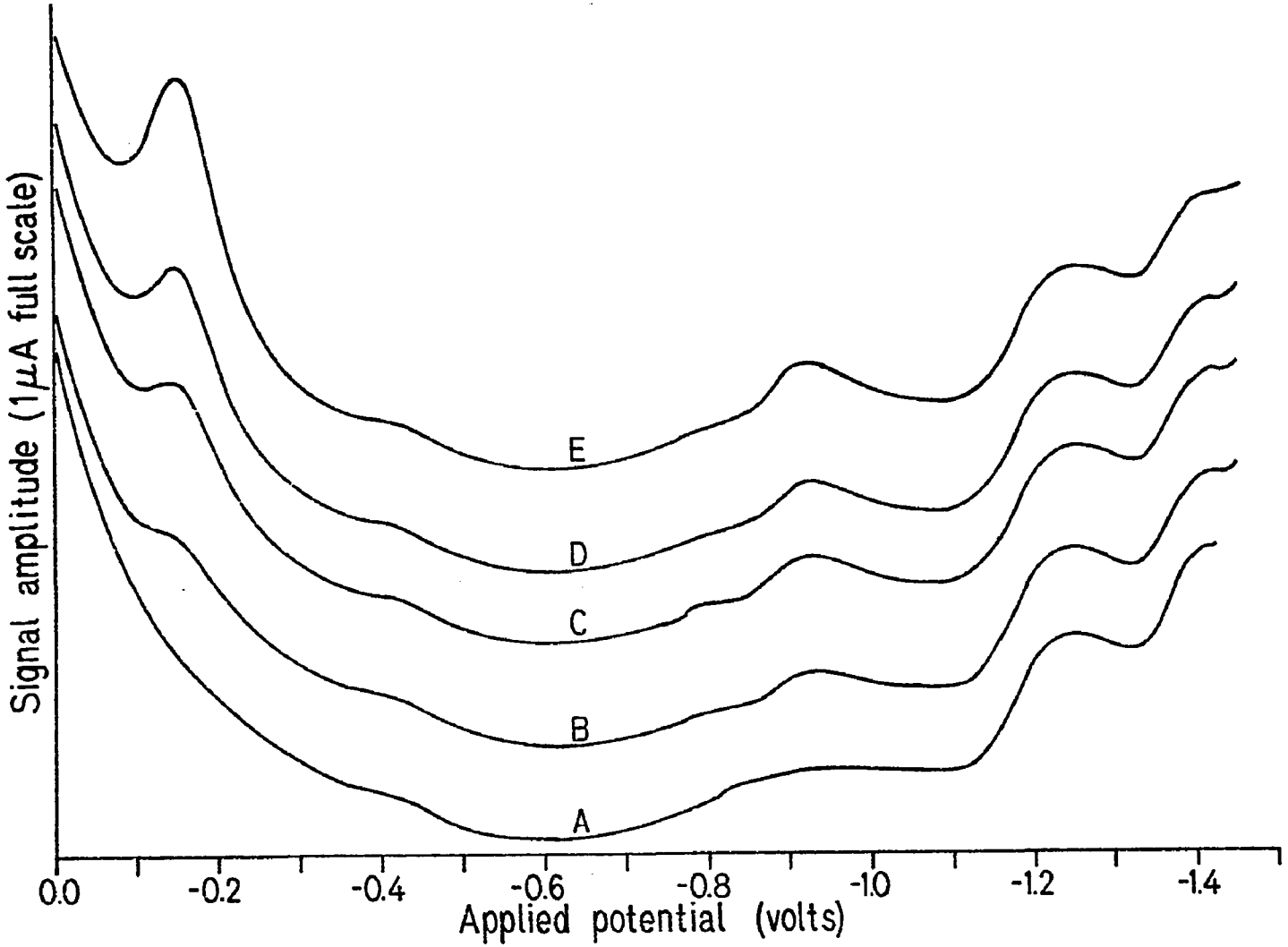
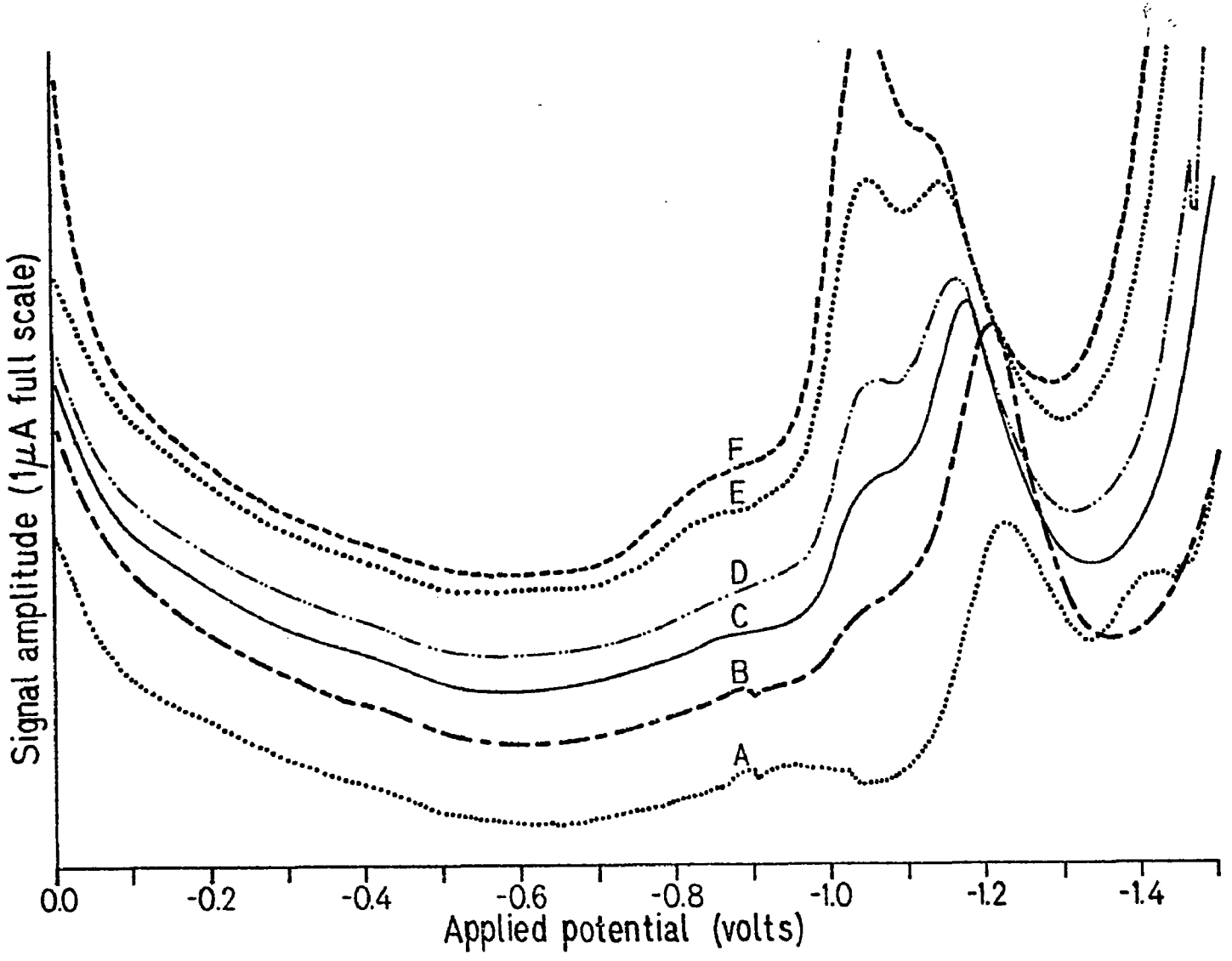

Signal amplitude (1 μA full scale)
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

Fig. 61. Polarographic interaction of Zn with the UB-M9 medium metabolite peak. 0.0 to -1.5 v scan range, 2 sec drop time, 1 μA full scale sensitivity.


Signal amplitude (1μA full scale)
F
E
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

The interaction of the metabolite peak with Ni was studied by titrating an aliquot of a 48 h incubated 2 X 10^{-5}M Ni UB-M9 culture (near end of lag) with Ni. The polarograms (Fig. 62) were quantitated by both peak height and peak area techniques, and the measured peak was compared to the results of the analysis of a set of uninoculated UB-M9 flasks containing a range of Ni concentrations. From the regression lines of that data the measured Ni from the height and area values of the titration was calculated. That data is presented in Table 39 and Fig. 62.

Two characteristics of the measured data should be noted. Firstly, although the curve of Ni added versus Ni measured was highly linear for both means of quantitation, in both cases the slope was less than one. Secondly, although the curve of peak area passed through the origin, the peak height curve intersected the x-axis at 1.25 X 10^{-5}M. The latter might indicate an excess metal binding capacity of 1.25 X 10^{-5}M in the medium sample.

To determine if the acidic metabolite of A. marinus might be gluconic acid or a derivative arising via an Entner-Doudoroff glucose oxidation pathway (Stanier et al., 1970), the polarographic effect of gluconic acid and 2-ketogluconic acid added to UB-M9 medium was examined. Although there was a definite polarographic effect (not shown), neither gave a peak of the same potential as the metabolite peak. Further, at an equivalent pH reduction the peak due to either of the gluconates was only a small fraction of the observed peak height of the metabolite at a growth point giving that pH

Fig. 62. Polarographic titration of a 45 h (late lag) culture of A. marinus grown in 2 X 10^{-5}M Ni UB-M9 with Ni to the following final concentrations: A) 2 X 10^{-5}M Ni, B) 3 X 10^{-5}M Ni, C) 4 X 10^{-5}M, D) 6 X 10^{-5}M Ni. Scan range 0.0 to -1.5 v, 2 sec drop time, 2 mv/sec scan rate, 2 μA full scale sensitivity.

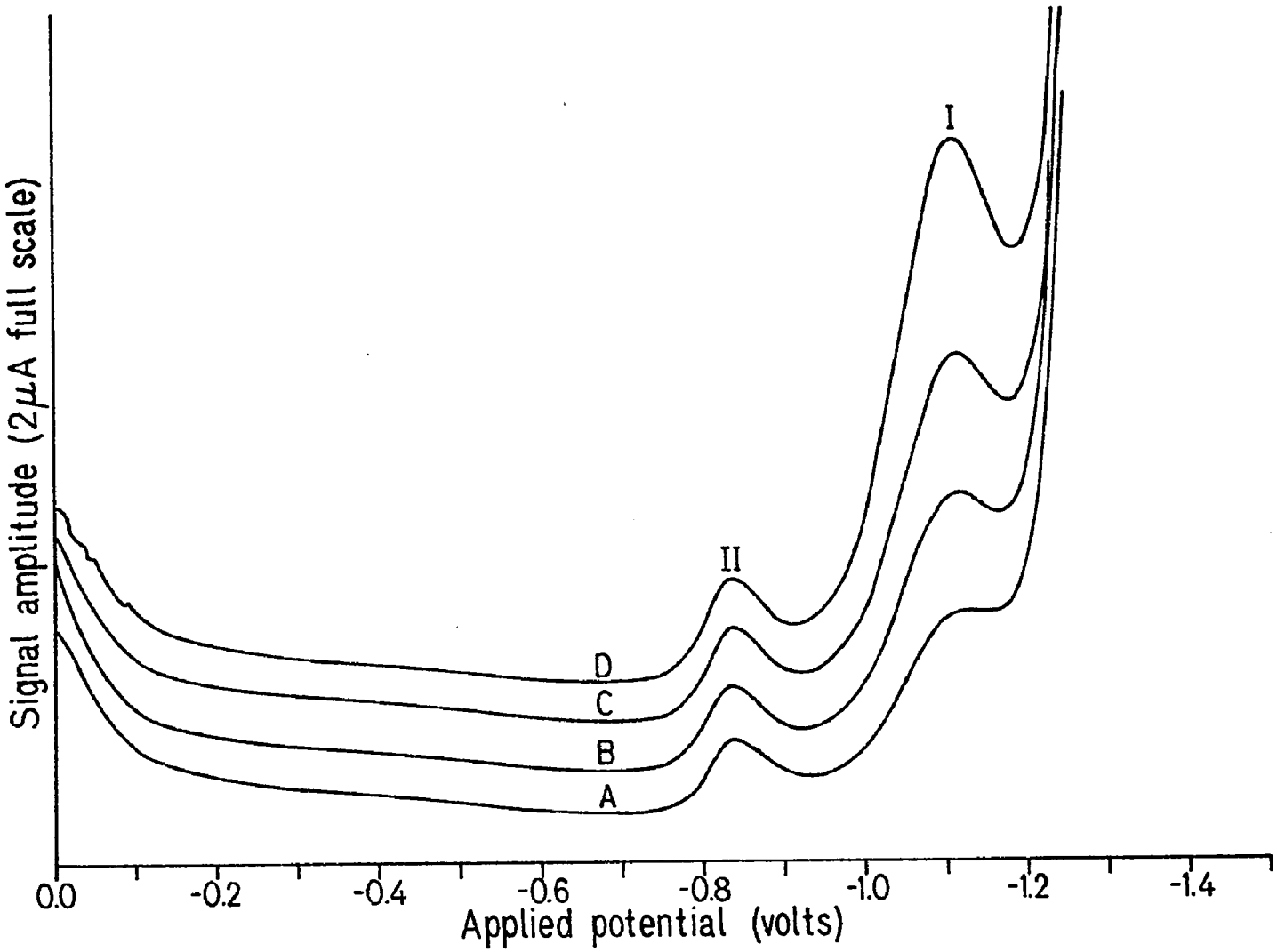

Signal amplitude (2μA full scale)
I
II
D
C
B
A
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

Table 39. Quantitation of UB-M9 medium Ni peaks.

A. Uninoculated medium

Ni, M	Peak potential, v	Peak height, mm	Peak area[a]
0	-	0	0
1×10^{-5}	-1.08	26	10.0
2×10^{-5}	-1.08	54	21.9
5×10^{-5}	-1.08	127	48.3
1×10^{-4}	-1.09	c.200	94.8

regression analysis		
	r=0.996	r=0.995
	$m=2.54 \times 10^{6}$	$m=9.41 \times 10^{5}$
	b=1.03	b=1.14

[a]arbitrary units; Dupont 310 curve resolver.

Table 39 Continued.

B. Inoculated medium

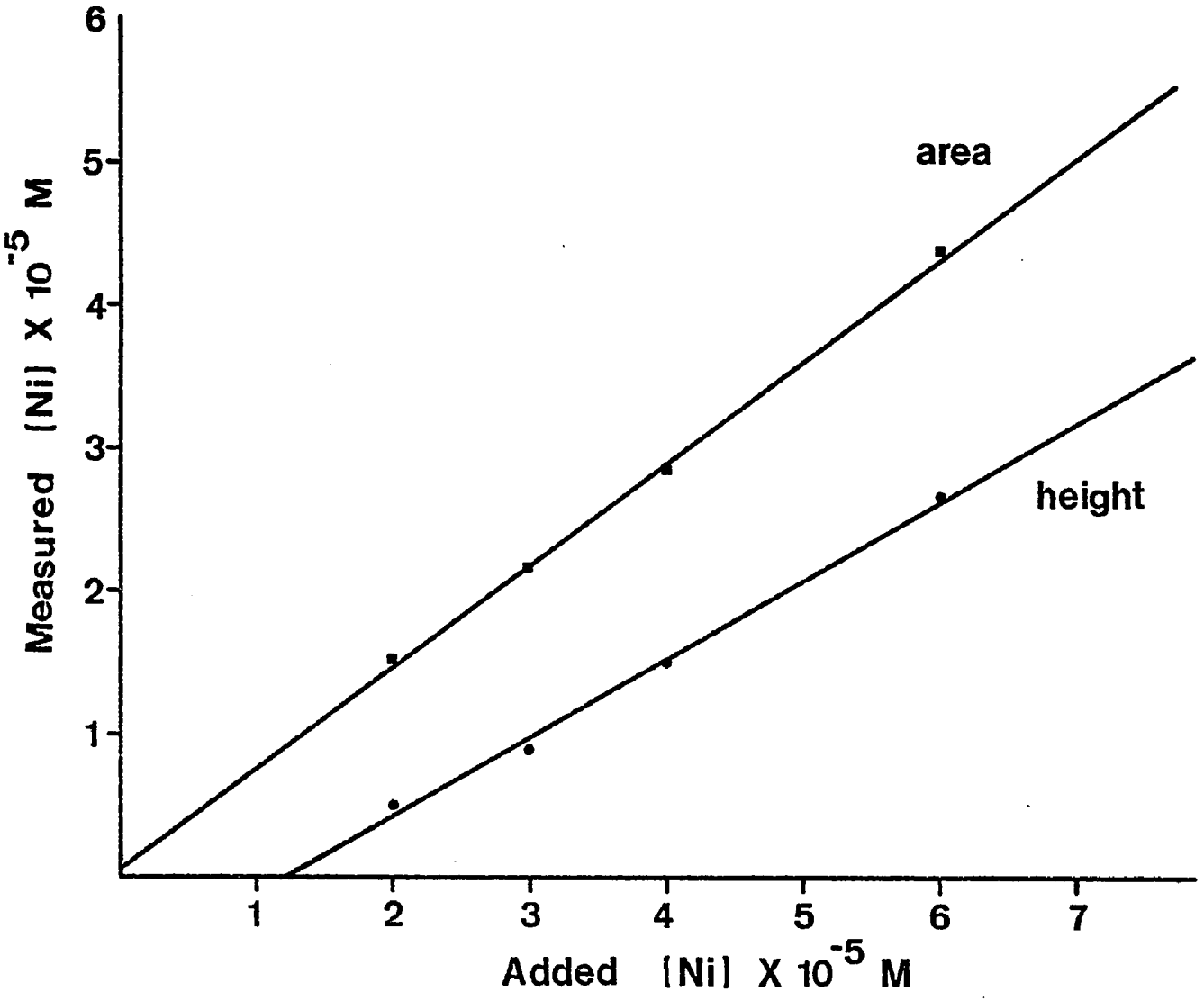
Ni, M[b]	Peak potential, v	Peak height, mm	Peak area[a]	/Ni/ Absolute M	/Ni/ measured[c] by: height, mm	area[a]
Initial (2×10^{-5})	-1.11	14	15.5	2×10^{-5}	5.11×10^{-6}	1.53×10^{-5}
$+ 1 \times 10^{-5}$	-1.11	24	21.6	3×10^{-5}	9.06×10^{-6}	2.17×10^{-5}
$+ 2 \times 10^{-5}$	-1.11	39	27.9	4×10^{-5}	1.50×10^{-5}	2.84×10^{-5}
$+ 4 \times 10^{-5}$	-1.11	69	42.3	6×10^{-5}	2.68×10^{-5}	4.38×10^{-5}
regression analysis					r=0.997	r=0.9989
					$m=5.52 \times 10^{-1}$	$m=7.15 \times 10^{-1}$
					$b=-6.7 \times 10^{-6}$	$b=4.71 \times 10^{-7}$

[a]arbitrary units; Dupont 310 curve resolver.

[b]Ni titration of a 48 h *A. marinus* UB-M9 culture with 2×10^{-5}M Ni.

[c]from regression formula in A.

Fig. 63. Quantitation of uninoculated UB-M9 medium Ni peaks by height and area.


Measured [Ni] X 10^-5 M
Added [Ni] X 10^-5 M
area
height
1
2
3
4
5
6
7

drop. The metabolite peak and the acidic metabolite was due to neither of the gluconic acids.

The polarographic response of metals is pH dependent (Spencer, personal communication). It was therefore undertaken to determine the effects of pH drops such as might occur during the lag phase of A. marinus in UB-M9 medium on the height, area, and potential of the Ni peak so that an appropriate correction could be applied to data derived from inoculated flasks. Samples of UB-M9 medium with 2 or 5 X 10^{-5}M Ni were placed into the polarographic cell into which was also placed a combination pH electrode through a spare port. The medium was titrated by dropwise addition of 0.1 N redistilled HCl, and the solution scanned polarographically at selected pH values (Table 40). There was a down shift of the peak potential of 20 to 30 mv as the pH dropped from 7.6 to 7.0. At the same time the peak height and area fell by 10 to 20 %.

With this data in hand, the polarographic effect of A. marinus growth was studied. Two sets of UB-M9 flasks were prepared, one with 2 X 10^{-5}M Ni and the other with 5 X 10^{-5}M Ni. Of 20 flasks at each Ni concentration 10 were inoculated (1.0 ml EQ, washed) and the other 10 kept as uninoculated controls. All flasks were placed on the shaking incubator and at intervals a pair of flasks, one inoculated and one control, was removed, the OD and pH measured (Fig. 64), and a polaragram run on each. The polarograms from the 5 X 10^{-5}M Ni growth set are shown in Fig. 65. The growth of the metabolite peak was already well off-scale by 27 h, c. 20 % through lag phase. The Ni peak shifted toward a slightly more negative potential,

Table 40. Quantitation of the pH effect on the Ni peak in uninoculated 2 and 5 X 10^{-5}M Ni UB-M9 titrated with 1 N HCl.

Ni, M	pH	Peak height, mm	%[a]	$E_{1/2}$[b]	ΔE	Peak area[d]	%[a]
2 X 10^{-5}	7.59	83	100	-1.08	0	50	100
	7.45	77	92.8	-1.09	-0.01	47.5	95
	7.22	74	89.1	-1.10	-0.02	44.5	89
	7.10	74	89.1	-1.10	-0.02	44.5	89
	6.94	71	85.5	-1.11	-0.03	43.0	86
	6.69	69	83.1	-1.11	-0.03	39.0	78
	6.06	-	-	-1.11	-0.03	43.5	87
	3.56	119[c]	(144)	-1.11	-0.03	45.0	90
5 X 10^{-5}	7.83	109	100	-1.09	0	100	100
	7.62	98	89.9	-1.10	-0.01	100	100
	7.43	92	84.0	-1.11	-0.02	95.2	95.2
	7.19	87	79.8	-1.12	-0.03	92.9	92.9
	7.03	83	76.1	-1.12	-0.03	90.0	90.0
	6.93	85	78.0	-1.12	-0.03	92.4	92.4
	6.58	85	78.0	-1.12	-0.03	92.4	92.4

[a]%pH of 7.59 for 2 X 10^{-5}M Ni and 7.62 for 5 X 10^{-5}M Ni.

[b]peak potential

[c]probably enlarged due to glucose peak ($E_{1/2}$=-1.33) merging into Ni.

[d]arbitrary units

Fig. 64. Growth as OD and pH changes of A. marinus in UB-M9 medium with 2 and 5 X 10^{-5}M Ni.

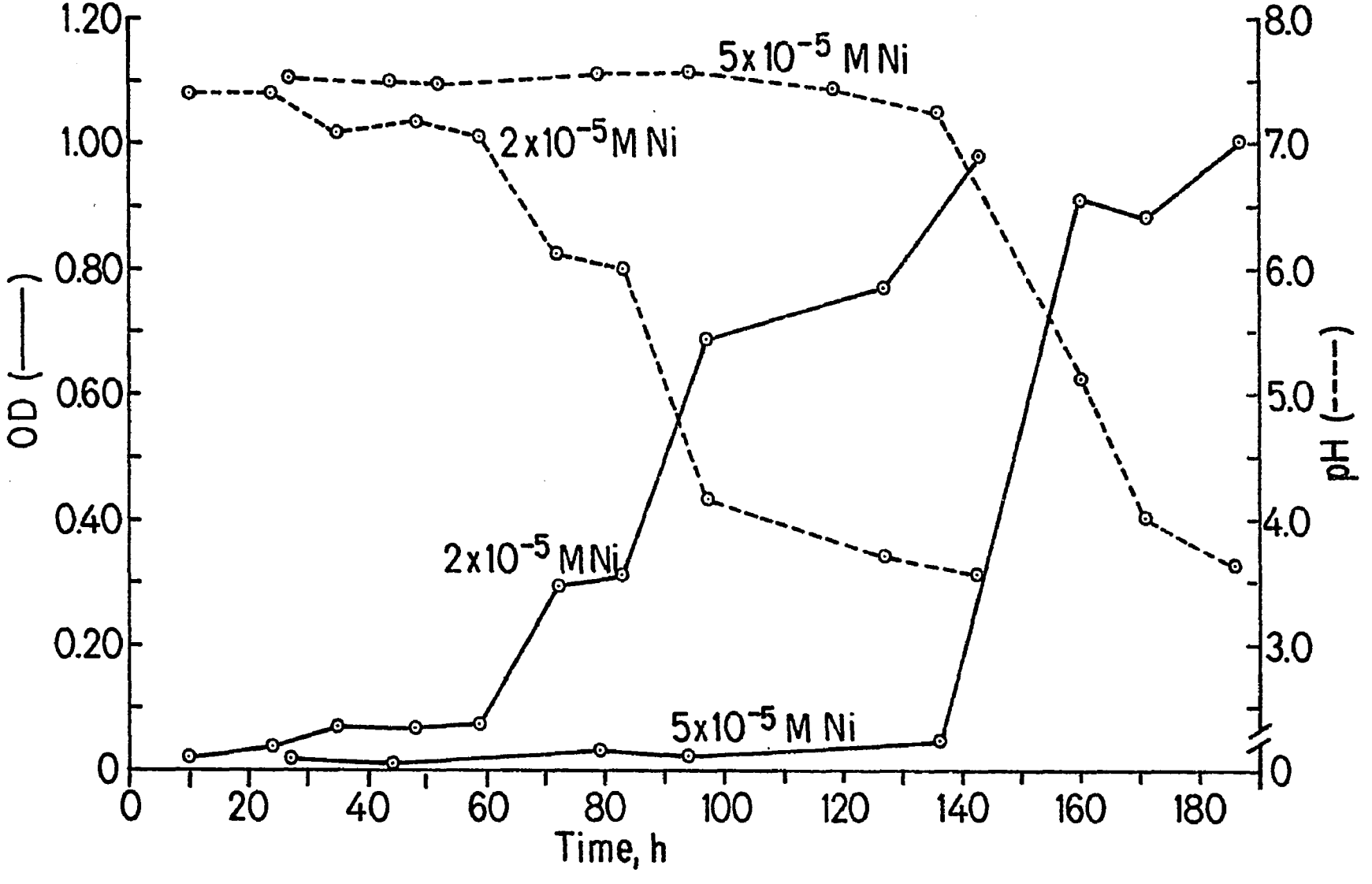
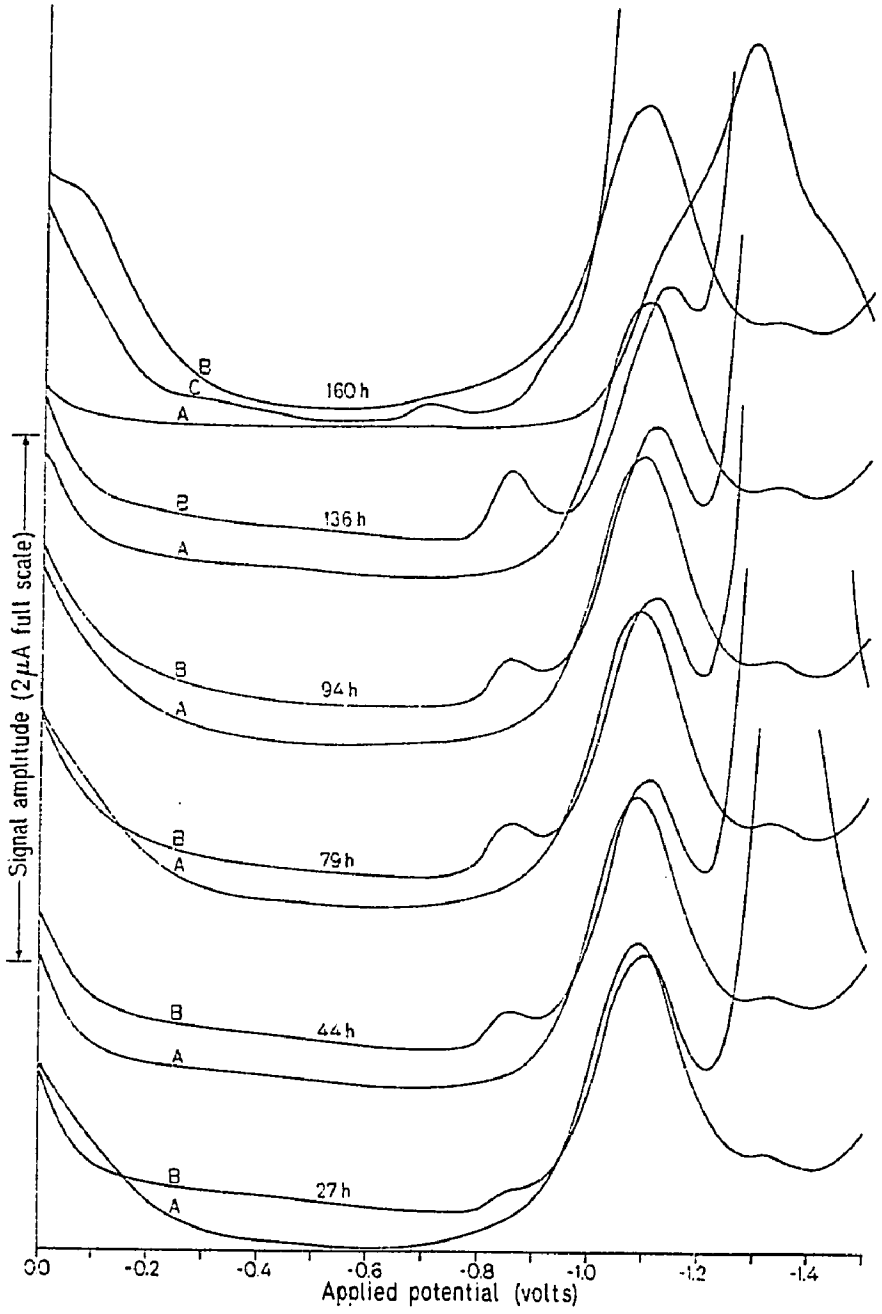

1.20
1.00
0.80
0.60
0.40
0.20
0
OD (——)
8.0
7.0
6.0
5.0
4.0
3.0
0
pH (- - -)
0
20
40
60
80
100
120
140
160
180
Time, h
5x10⁻⁵ M Ni
2x10⁻⁵M Ni
2x10⁻⁵ M Ni
5x10⁻⁵ M Ni

Fig. 65. Polarographic changes during the growth of A. marinus in UB-M9 medium, 5 X 10^{-5}M Ni. Scan range 0.0 to -1.5 v, 2 sec drop time, 2 mv/sec, 2 μA full scale sensitivity. A. uninoculated control flask. B. inoculated culture.


B
C
A
160 h
B
A
136 h
B
A
94 h
B
A
79 h
B
A
44 h
B
A
27 h
Signal amplitude (2 μA full scale)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Applied potential (volts)

and the height decreased relative to its baseline. As the incubation time increased, the relative size of the Ni peak continued to decrease. The off-scale metabolite peak continued to increase, gradually encroaching onto the Ni peak which it had obliterated by about the end of lag phase. A third peak at -0.85 v appeared and increased in size. This third peak may have been another metabolite.

Results from the quantitation of the Ni peaks are presented in Table 41. These data were for measurements by the peak height technique only. Attempts to use the curve resolver failed as it was impossible to accurately set the curve shape of the far off-scale metabolite peak and without this information the operator could choose virtually any Ni peak area by very minor manipulations. The data for the calculated molarity of free Ni (corrected for the pH effect) is presented in Fig. 66. The net peak potential change (corrected for pH shift) is presented in Fig. 67. There was a consistent drop both of the height of the Ni peak and of the free Ni molarity during the lag phase of the organism in both media. In 2×10^{-5}M Ni the free Ni molarity dropped to 3.7×10^{-6} by the end of lag phase; in 5×10^{-5}M Ni the free molarity dropped to 2×10^{-5}M by the end of lag phase. Especially in the case of 2×10^{-5}M the rate of decrease was not linear with time. This may reflect the same process seen in the medium conditioning experiment (Table 24) in which the rate of lag reduction was greatest during the first part of the lag phase. For both Ni concentrations (Fig. 67) there was an

Table 41. Quantitation of polarographic changes in Ni peak of 2 and 5 X 10^{-5}M Ni UB-M9 medium during lag phase of A. marinus.

2 X 10^{-5}M Ni

	Peak height					Peak potential				
Time,h	uninoc control	pH[a] factor	inoc	% C[b]	EQ molarity	uninoc control	inoc	$E_{1/2}$	$E_{1/2}$ due to pH	net E
10	83	1.08	50.8	61.2	1.2 X 10^{-5}	-1.09	-1.10	-0.01	-0.01	0.00
35	74	1.12	16.8	22.7	0.45 X 10^{-5}	-1.09	-1.12	-0.03	-0.02	-0.01
48	82	1.12	17.9	21.8	0.44 X 10^{-5}	-1.09	-1.12	-0.03	-0.02	-0.01
59	84	1.12	15.7	18.7	0.37 X 10^{-5}	-1.09	-1.12	-0.03	-0.02	-0.01
72[c]	84	-	-	-	-	-1.08	-	-	-	-

5 X 10^{-5}M Ni

0	109	-	-	-	-	-1.09	-	-	-	-
27	119	1.12	86.2	72.4	3.6 X 10^{-5}	-1.09	-1.10	-0.01	-0.015	0.00
44	115	1.16	80.0	69.6	3.5 X 10^{-5}	-1.08	-1.11	-0.03	-0.015	0.015
52	113	1.17	77.2	68.3	3.4 X 10^{-5}	-1.08	-1.10	-0.02	-0.02	0.00
79	118	1.12	70.6	59.8	3.0 X 10^{-5}	-1.08	-1.11	-0.03	-0.01	0.02

Table 41 Continued.

Time, h	uninoc control	pH[a] factor	inoc	% C[b]	EQ molarity	uninoc control	inoc	$E_{1/2}$	$E_{1/2}$ due to pH	net E
94	114	1.11	69.9	61.3	3.1×10^{-5}	-1.08	-1.11	-0.03	-0.01	0.02
118	110	1.19	58.3	53.0	7.6×10^{-5}	-1.08	-1.11	-0.03	-0.02	0.01
136	108	1.23	44.2	40.9	2.0×10^{-5}	-1.08	-1.13	-0.05	-0.025	0.025
160[d]	(118)	-	20	-	-	(-1.08)	-1.14	-	-	-

[a]corrected for pH peak size change (Table 40).

[b]% of control

[c]At 72 h and above Ni peak "swallowed" by metabolite.

[d]Ni difficult to read as it appeared as 'bump' on metabolite peak.

Fig. 66. Changes in the equivalent molarity of free Ni during lag phase (Fig. 65) of A. marinus in UB-M9 medium with 2 or 5 X 10^{-5}M Ni.

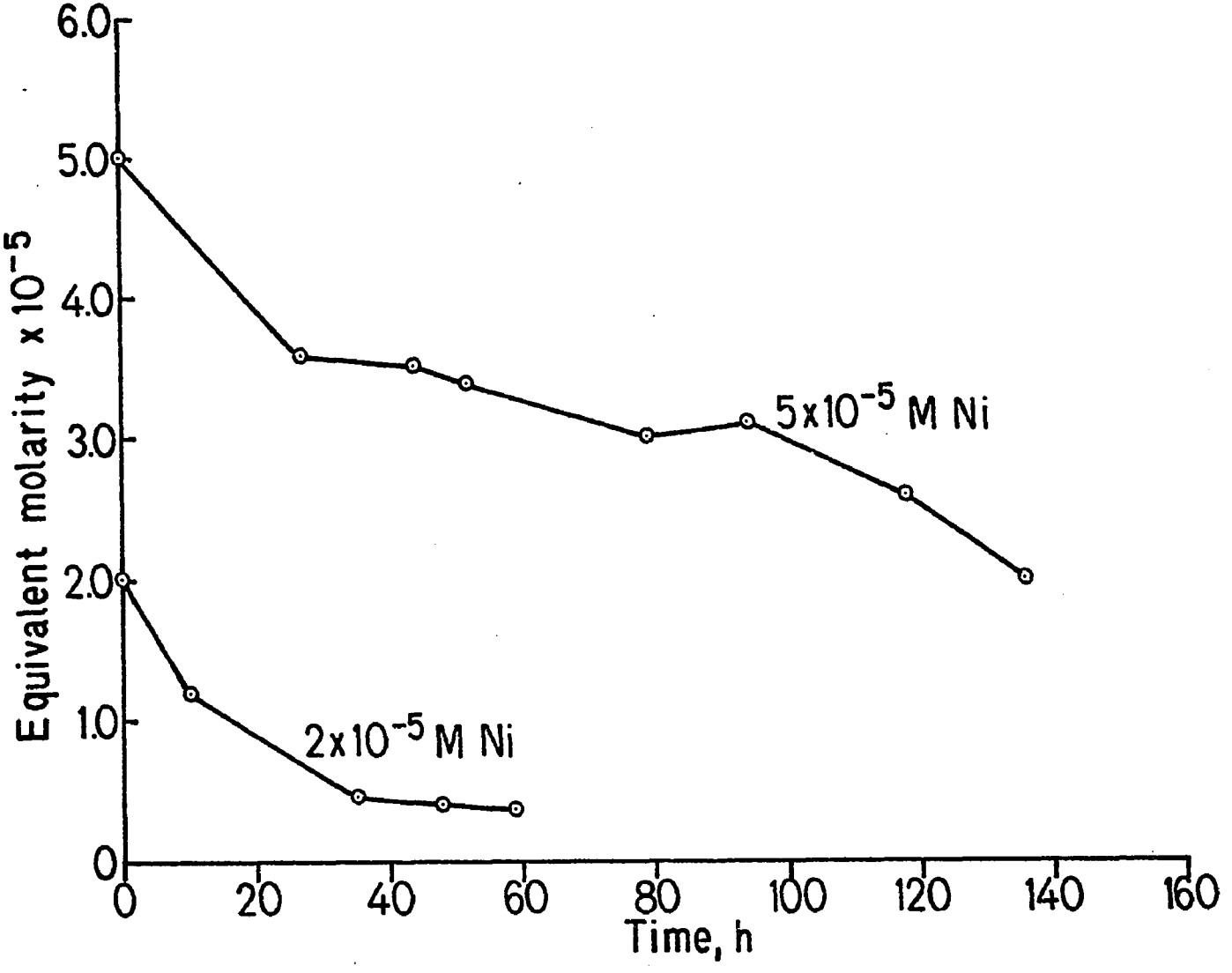
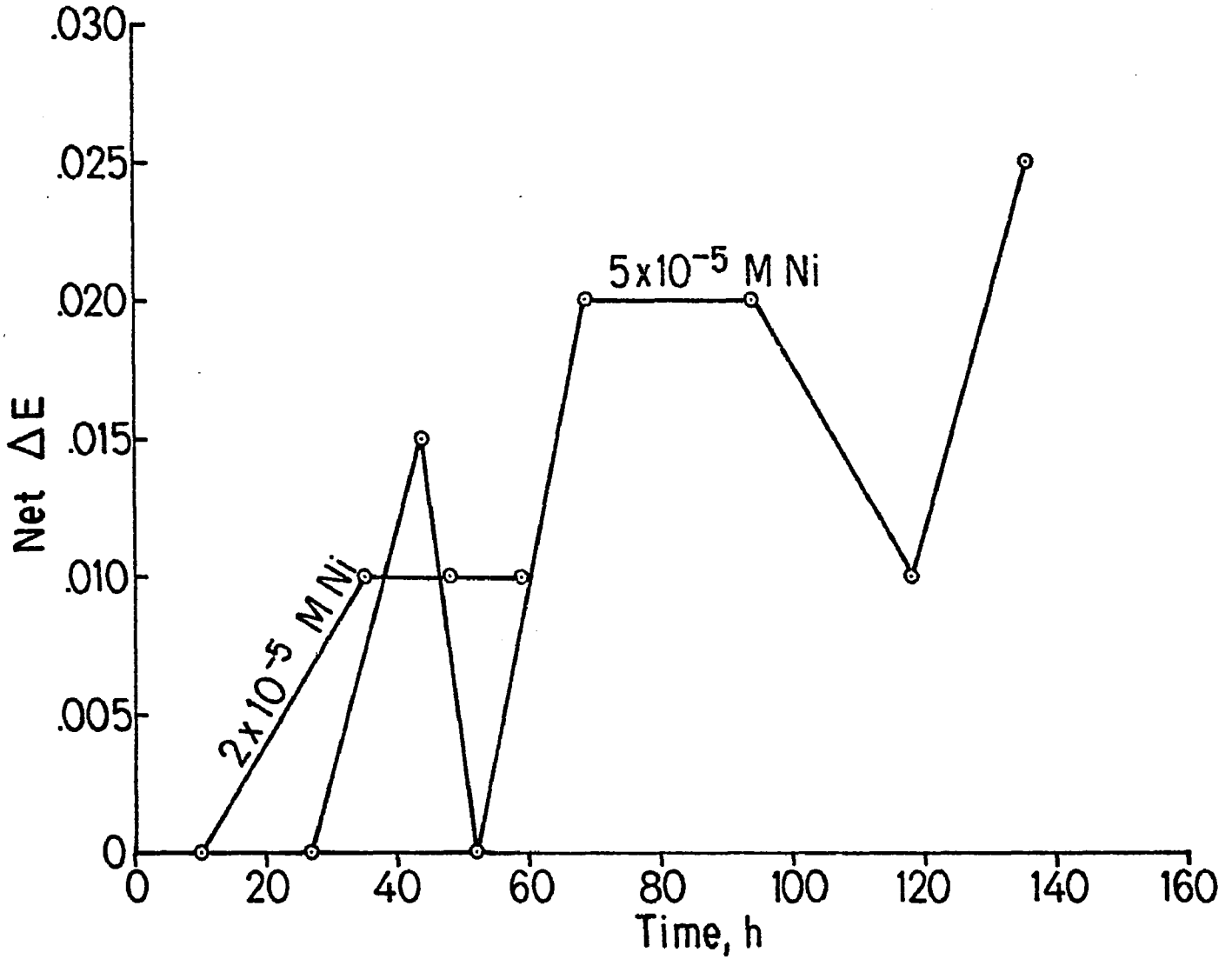

6.0
5.0
4.0
3.0
2.0
1.0
0
Equivalent molarity x 10⁻⁵
5x10⁻⁵ M Ni
2x10⁻⁵ M Ni
0
20
40
60
80
100
120
140
160
Time, h

Fig. 67. Changes in the net $E_{1/2}$ of the Ni peak during lag phase (Fig. 65) of A. marinus in UB-M9 medium with 2 or 5 X 10^{-5}M Ni.


.030
.025
.020
.015
.010
.005
0
Net ΔE
5x10⁻⁵ M Ni
2x10⁻⁵ M Ni
0
20
40
60
80
100
120
140
160
Time, h

upward potential change. The higher Ni concentration gave greater potential shifts.

As was noted in the section on the polarographic effect of known chelating agents, a metal complex may have two effects polarographically. Either the peak size will be decreased relative to the free metal or the peak will be shifted toward a more negative value. It would appear that both mechanisms were functioning in this case.

During the lag phase of A. marinus in UB-M9 medium, there were changes of the speciation of Ni as detected by differential pulse polarography which were interpreted as being caused by the formation of Ni complexing organic compounds by A. marinus. These metal binding agents were considered to be the mediators of the medium conditioning effect by which A. marinus overcame the Ni-induced lag phase which was characteristic of its reponse to toxic concentrations of Ni in UB-M9 medium.

CHAPTER V

DISCUSSION

A. Taxonomy

The genus Arthrobacter was established by Conn and Dimmick (1947) to differentiate a group of soil coryneform bacteria which were non-acid fast, non-fastidious, and showed a distinct cycle of morphology and gram staining reaction during growth. Cells of Arthrobacter are coccoid and gram positive in old cultures. Upon inoculation into fresh medium the coccoid cells grow out into gram negative rods which are irregular, showing curved and club shapes, and V - formations due to snapping post-fission movement of dividing cells. During late exponential phase and stationary phase cells shorten, ultimately into cocci, and the gram reaction becomes first variable, then positive (Keddie, 1974). The Arthrobacter species are non-motile or motile by a few lateral flagella and are physiologically non-distinctive. Arthrobacter occurs in a variety of terrestrial habitats in addition to soil (Mulder et al., 1966) and may be prominent in aquatic environments (Sieburth, 1964; Dubinina and Zhdanov, 1975).

The results of gram stains, observations of cellular morphology and biochemical tests indicate that the organism identified as Arthrobacter marinus by Cobet, Wirsen, and Jones (1970) does not meet the specifications of that genus. Arthrobacter marinus was clearly gram negative in all phases of growth

(Fig. 2 b, c). The pattern of cell morphology changes observed in the Arthrobacter marinus growth cycle was much less distinctive than that of Arthrobacter. Furthermore, the change of cell morphology from rods in young cultures to cocci or coccobacillary rods in older cultures is not limited to Arthrobacter, indeed it occurs in marine Vibrios (Felter, Colwell and Chapman, 1969; Novitsky and Morita, 1976), a variety of soil bacteria (Balkwill, 1977), and even in E. coli (Chesbro, personal communication). The difference in the cell content of lipopolysaccharide, as detected by the Limulus amebocyte lysate test was confirmatory of the gram stain results, indicating that Arthrobacter marinus has a gram negative-type cell wall structure. This can be seen in the electron micrographs of Arthrobacter marinus by Cobet et al. (1971) which show a typical "double track" gram negative cell wall structure. Also of note is the total lack of sensitivity of Arthrobacter marinus to 0/129 antibiotic while known Arthrobacter strains were highly sensitive (Table 7). The potential exists that 0/129 sensitivity could be of use as a differential characteristic for the genus Arthrobacter. While 0/129 is most familiar as a Vibrio-specific agent (Collier, Campbell, and Fitzgerald, 1950; Shewan, Hodgkiss, and Liston, 1954), it was originally published as an inhibitor of gram positive bacteria such as Streptococcus (Daniel et al., 1947).

There can be little doubt that the organism deposited with the ATCC as P. marina, and the four strains of Arthrobacter marinus are closely related (Table 8). Two studies (current; Baumann et al., 1972), using different arrays of tests

(Colwell and Wiebe, 1970; Baumann et al., 1972) reached identical conclusions as to the relatedness of organisms independently isolated in different oceans. This was a striking demonstration of the consistency of Adansonian Taxonomy. The major disagreement between the Baumann et al. (1972) results and current findings was the question of flagellation. Current results were in substantial agreement with other workers (Table 9), although interpretation of subjective tests differed. Similarly, within current results, P. marina differed from Arthrobacter marinus on a few tests (Table 6) such as colony shape and wet mount motility on which subjective judgement was required.

If Arthrobacter marinus is not properly a member of that genus, where should it be placed? The assignment of this organism to Pseudomonas is questionable on several grounds other than flagellation, as it was oxidase negative, arginine dihydrolase negative, and lacked fluorescent pigments, all characteristically positive in Pseudomonas though not strictly differential traits for the genus (Doudoroff and Palleroni, 1974).

The mixed polar and petritrichous flagellation shown by Arthrobacter marinus was differentiated from a pattern shown by Vibrio (Shinoda et al., 1974; Deboer, Golten and Scheffers, 1975), Chromobacterium (Sneath, 1956), Aeromonas (Leifson, 1963), Pseudomonas stutzeri (Palleroni et al., 1970), and Beneckea and Photobacterium (Allen and Baumann, 1971). In all those organisms the primary flagellation was by a single, sheathed, polar flagellum. That is the pattern in liquid culture. When

grown on agar surfaces the cells had, in addition to the polar flagellum, multiple lateral unsheathed flagella of shorter wavelength than the polar flagellum. This definitely was not the pattern of Arthrobacter marinus. There was no evidence for sheathed flagella, nor were flagella of different wavelengths seen, nor was there a difference in the flagellation of Arthrobacter marinus on liquid or solid medium.

The use of flagellation as a major taxonomic character has been the subject of a number of critiques (Conn and Elrod, 1947; Leifson, 1960; 1966), emphasizing the role which observer bias and/or error can have in the assignment of flagellation type. It is, for instance, typical to find individual cells in a peritrichous population which show polar flagellation (Leifson, 1966; Hodgkiss, 1960). The major (sometimes only) means of differentiation between Pseudomonas and Alcaligenes is the pattern of flagellation: Pseudomonas is polar, Alcaligenes is lateral or peritrichous. Stanier (1976), in reviewing the taxonomy of Pseudomonas, has emphasized the weakness inherent in the use of flagellation as the character separating these two genera.

As the gram negative oxidative rod-shaped bacteria are currently divided (Doudoroff and Palleroni, 1974), a peritrichous organism cannot be placed into Pseudomonas. When the genus Hydrogenomonas was revised (Davis, Doudoroff, and Stanier, 1969), the similar rod-shaped members of the illigitimate genus was divided into two groups: one went into Pseudomonas and the other went into Alcaligenes with flagellation the primary difference between the two groups. When the genus

Achromobacter was rejected, the motile strains were reassigned to the genera Pseudomonas or Alcaligenes on the basis of flagellation (Hendrie, Holding, and Shewan, 1974). Photomicrographs of Alcaligenes species closely resembled the optical and electron microscopal appearance of flagellated Arthrobacter marinus cells (Leifson, 1960; Aragno et al., 1977).

In addition to the genus Alcaligenes, other possibilities for classification exist among the oxidative, peritrichous, gram negative rods. The ability of the organism to grow on Ashby's N-free medium might indicate membership in the family Azotobacteriaceae. Although the growth on Ashby's medium was repeatably transferable, it was very weak. This observation combined with the absence of characteristic cell and colonial morphology excluded this classification.

As was emphasized by Holding and Shewan (1974) in the Bergey's Manual discussion of Alcaligenes, the two genera of the family Rhizobiaceae (Jordan and Allen, 1974) do not significantly differ from Alcaligenes, except that both Rhizobium and Agrobacterium induce cortical hypertrophies (crown galls and root nodules) in a variety of plant species. An extensive series of Agrobacterium species has been reported from marine sources (Ahrens, 1968). However, the description of Arthrobacter marinus did not match the brown pigmented, rosette-forming organisms of Ahrens. Further, it is questionable whether those bacteria form a natural taxomonic grouping, and if so, whether they belong in Agrobacterium. It has been emphasized by Jordan and Allen (1974) and Holding and Shewan (1974) that in the absence of plant inoculation tests placement

of a candidate organism into either of the Rhizobiaceae genera was difficult. Owing to the high degree of host-symbiont (-parasite) specificity shown by these organisms, it is impossible to place members isolated from sources other than infected hosts without performing inoculation tests on many potential host species.

I propose placement of the cluster of strains composed of P. marina, Arthrobacter marinus, and related strains into genus Alcaligenes as A. marinus. The organism matched the current description of the genus (Holding and Shewan, 1974; Hendrie, Holding, and Shewan, 1974) with the exception of oxidase (Kovac's) reaction. While the absence of alkaline reaction in litmus milk (hence the name Alcaligenes) may seem a major failing, of the four species listed in Bergey's Manual (Holding and Shewan, 1974), only A. faecalis was listed as giving an alkaline milk reaction. The production of acid from carbohydrate, as in A. marinus, was shown by A. paradoxus. The combination of litmus milk reaction, utilization of inorganic nitrogen, failure to grow chemolithotrophically on H_2, and failure to hydrolyze starch and gelatin differentiate A. marinus from other described species of the genus.

The pathways and regulation of cellular catabolism of a group of marine bacteria including P. marina, marine Alteromonas sp., and marine Alcaligenes sp. have been investigated (Baumann and Baumann, 1973; 1974; Sawyer, Baumann and Baumann, 1977). These regulatory pathways may have taxonomic implications. Five classes of aspartate kinase activity regulation were found (Baumann and Baumann, 1974). P. marina

had similar regulation to A. cupidus, A. pacificus, A. venustus, and A. aestus, but differed from other Pseudomonas and Alteromonas species. This evidence was thus corroboratory of my results with A. marinus.

The megalomorphic response of A. marinus may also have taxonomic implications. Reports of cell morphology effects resembling A. marinus, which are rare, have been cited in the Literature Review. Gonye (1972) tested several soil coryneform bacteria which showed no megalomorphic response to Ni. Further, while A. marinus gave abnormal morphology due to other metals, only Ni caused the extremely enlarged, vacuolated cells (Cobet, 1968). Though independently isolated from different oceans, P. marina and A. marinus gave identical megalomorphic responses to Ni. Of Ni-resistant estuarine bacteria, 70 % (Table 11) gave megalomorphic cells due to Ni. None of 230 estuarine isolates resistant to Pb, Co, MoO_4^-, or Hg (but not tested for Ni-resistance) clustered with A. marinus by numerical taxonomy (Austin et al., 1977; Austin, personal communication). The potential thus exists that Ni-resistant marine bacteria, and more exactly the megalomorph-producing Ni-resistant marine bacteria, form a single taxonomic grouping.

B. Ni Toxicity Response

The toxicity of a metal to a microorganism is effected by a variety of factors including the concentration of antagonistic ions (Abelson and Aldous, 1950), the differences in the intrinsic resistance of the organism (Avakyan, 1967; Babich and Stotzky, 1977a; Golubovich, 1974), and the speciation of the

metal. In the current studies all experiments were performed in artificial seawater solutions; there were thus adequate and constant concentrations of the antagonistic cations (primarily Mg) present. Similarly, as all studies were conducted with the same organism, variations in intrinsic sensitivity were removed. Thus the effect of metal ion speciation was isolated. Based on the data reported in the literature and my experimental results, a single model of metal ion toxicity will be demonstrated. Metals chelated by strong metal-binding agents such as EDTA are nontoxic. Free metal ions and metal complexed by sufficiently weak inorganic or organic ligands are toxic at a concentration characteristic of the cell. Complexing agents of intermediate stability will be toxic if the stability constant of the complexing agent is less than that of the metal ion target site of the cell.

Furthermore, the cell is not necessarily a passive agent. Depending on the organism and the toxic metal, the cell may either react by modifying itself such that its intrinsic sensitivity is altered (cell "adaptation"), or the cell may alter the external state of the metal by production of complexing agents or physical removal of the metal ions. Evidence will be presented to demonstrate the modification of the speciation of Ni as a mode of metal resistance of A. marinus when grown in a non-complexing medium.

Metal chelating and complexing agents ameliorate the toxic effects of heavy metals to microorganisms (Sadler and Trudinger, 1967; Pratt, 1953; Martell and Calvin, 1952; Shaw, 1961). These observations have led to the proposal that the

agent of metal toxicity was the free ion and that metal ions complexed by organic ligands were rendered nontoxic (Sadler and Trudinger, 1967; Cobet, Wirsen, and Jones, 1970). Heavy metals were 100 to 1000 fold more toxic in defined composition than in peptone media (Schade, 1949), and amino acids were effective chelating agents (Albert, 1950). Thus, in such complex media the metal concentration at which metal toxicity appeared was proposed to be that at which the metal binding capacity of the medium was exceeded and free metal ion appeared in solution (Cobet, 1968; Jones, Royle, and Murray, 1976; Sadler and Trudinger, 1967).

Analysis of the speciation of Ni at the concentration at which metal toxicity to A. marinus in 2216E LN medium appeared was attempted by pulse polarography. There was no distinct change of the polarograms (Fig. 41) which correlated with the appearance of metal toxicity (c. 1 X 10^{-4}M; Fig. 30).

The Chelex-100 chromatography technique applied to 2216E LN medium gave evidence that the speciation of Ni in that medium could not be interpreted as purely a strong metal binding phenomenon. There was a significant fraction of Ni at metal concentrations well below the A. marinus toxicity threshold which was "free" as defined by the technique (Fig. 56). This indicated that the peptone and yeast extract components of the medium had a wide variety of metal complexing ligands of widely varying metal avidities, as predicted by the variation of the stability constants of constituent amino acids (Sillen and Martell, 1964; 1971). Furthermore, the ligands were not at equilibrium with the metal, even after

autoclaving. In equilibrium, at low metal concentrations the metal ions would have been in the form of the strongest ligands. By contrast, in 2216E LN medium there was Ni both as strongly bound (Chelex-100 unextractable) and as weakly bound or free forms (Chelex-100 extractable), even at Ni concentrations several orders of magnitude lower than the Ni toxicity threshold.

In the case of 2216E LN medium the agent of metal toxicity thus was not free metal ion *per se*, but rather weakly complexed metal ion. Various components of the bacterial cell, proteins, nucleic acids (Wacker and Valee, 1959), and cell wall components (Beveridge *et al.*, 1976); were capable of greater or lesser metal ion binding (Bowen, 1966). I propose that the point at which toxicity appeared in 2216E LN medium was the Ni concentration at which those medium ligands of metal-binding stability greater than those of essential metabolic or structural components of *A. marinus* cells became saturated, allowing the metal target sites of the cells to compete for the metal. It should thus be possible to determine the effective stability of the Ni target by using Ni complexing agents of selected stability constants as competitors in a medium free of other complexing agents.

The growth response of *A. marinus* in TRIS-M9 medium gave further evidence that free Ni ion was not the agent of metal toxicity. Tris buffer, while weaker than EDTA, is a moderately avid chelating agent (Anderson and Morel, 1978). Thus for Cu (Ni stability constants are essentially identical to Cu) EDTA has a pK_m of 18.0, Tris 11.1, and histidine 8.8

(Sillen and Martell, 1964; Bai and Martell, 1969, as quoted in Anderson and Morel, 1978). Anderson and Morel (1978) calculated that in a medium 1 X 10^{-3}M in Tris, an added Cu concentration of 3.2 X 10^{-5}M gave a calculated Cu ion activity (free Cu) of 7.9 X 10^{-10}M. Thus TRIS-M9 medium, which was 4.13 X 10^{-2}M in Tris, would have essentially no free Ni at Ni concentrations of 1 X 10^{-3}M or greater. While Ni at the concentration ranges over which inhibition occurred in TRIS-M9 (5 X 10^{-5} to 5 X 10^{-3}M) was thus predicted to be completely complexed by TRIS-M9, it exerted toxic effects on A. marinus at concentrations 400 times lower than the medium chelating-agent concentration.

If the Ni toxicity level in 2216E LN was governed by competition between cell receptors and medium ligands, it would then be expected that in UB-M9 medium Ni toxicity would occur at a much lower concentration due to the lack of competing ligands. This phenomenon is seen in the inhibition of Proteus by Co; in defined medium the inhibition threshold dose is reduced by 400 fold in defined, non-complexing medium compared to complex medium (Schade, 1949). By contrast, the threshold inhibitory dose of Ni to A. marinus in UB-M9 was only 10 to 20 fold less than in 2216E LN (Fig. 11 versus Table 19). This would imply that in the absence of added complexing agents the toxic threshold level of metal ion is determined by characteristics of the species.

The effects of the chelating agents EDTA and histidine indicated that they quantitatively reversed the toxic effects of Ni on A. marinus, especially EDTA (Table 33). Ni complexed

by EDTA in a 1:1 ratio was completely non-toxic. In the case of histidine, there was also a quantitative supression Ni toxicity, with a 2:1 or 3:1 Ni to ligand ratio indicated. The occurrence of a 1:1 Ni-EDTA chelate is well documented (Bell, 1977; Dwyer and Mellor, 1964), but a structure by which histidine complexed three equivalents of metal was not found. Histidine has four coordination sites which allow a single molecule to complex two Ni ions (Bell, 1977). The report of Schade (1949) indicated detoxification of Co by histidine at a 2:1 molar ratio.

It was difficult to reconcile the quantitative suppression of Ni toxicity by histidine (Table 33), while Tris buffer (a slightly more avid chelator than histidine) did not. There were two possible explanations. Firstly, the cell systems which were inhibited by free Ni at a 1 X 10^{-5}M concentration may be different from those effected by Tris- or peptone-bound Ni at a 1 to 2 X 10^{-4}M concentration. Secondly, Tris was not acting toward Ni in a manner similar to EDTA or histidine. Rather, it may have been saturated by the major ions of the seawater medium.

There are differing reports concerning whether organically complexed metal ions are toxic. Using yeasts and fungi, Russian workers have indicated that free Cu and Pb or those metals complexed by weak complexing agents (such as monofunctional organic acids which cannot form chelate bonds) were toxic, whereas the metals complexed by stronger ligands (amino acids and bifunctional organic acids which form chelate bonds) were nontoxic (Avakyan and Rabotnova, 1966; Zlotchevskaya

and Rabotnova, 1967; Avakyan, 1971; Avakyan and Rabotnova, 1971). Similar results were reported in marine phytoplankton in which the toxicity of Cu was a function of the free cupric ion activity (Sunda and Guillard, 1976; Anderson and Morel, 1978).

By contrast, bound metal ion can act to inhibit microorganisms. The enhanced metal toxicity caused by such compounds as 8-hydroxyquinoline has been previously mentioned. The form of Hg in bacterial growth media was electrochemically characterized by Ramamoorthy and Kushner (1975). They found that in peptone media, free Hg was not detectable, even though the Hg concentration was strongly inhibitory to the majority of bacteria isolated from a river. Similarly, Cu caused inhibition of Chlorella and natural phytoplankton populations at Cu concentrations below that at which free Cu ion was detectable by electrochemical titration. Only when EDTA was added did the threshold of metal toxicity match the point at which free metal ion appeared.

I concluded that these differing reports arise from differences both between the avidities of the target sites for different metals in a single organism, and for different organisms for a single metal. Thus the report of Ramamoorthy and Kushner (1975) can be attributed to very high avidity Hg binding sites, able to compete with EDTA for Hg ion, such as amino acid sulfhydryl groups, while the reports of Avakyan and co-workers would indicate that the Cu and Pb targets of the fungi studied were rather weak, and only able to compete with weak complexing agents. I concluded that A. marinus must

have Ni ion target sites of lower avidity than histidine (pK_m=8.8) but higher than the ligands present in UB-M9 (glucose and inorganic anions, pK_m 2.0).

Additional information concerning the interaction of Ni with A. marinus was derived by examination of the uptake of the metal by cells. If the hypothesis above concerning metal target site binding avidities was valid, it would follow that in a medium such as 2216E LN in which medium ligands successfully compete with the cell for metal, uptake would be reduced. This was the case. There was no quantifiable Ni uptake by A. marinus from 2216E LN. The use of heavy cell suspensions and high specific activities of ^{63}Ni allowed the quantitation of Ni uptake by A. marinus in UB-M9 (Table 37). Uptake was low, non-energy dependent, and slow. These results were confirmed by Bartha and Atlas (1972) who found no detectable Ni uptake by A. marinus in contrast to nearly quantitative removal of 1.5 X 10^{-7}M Ni from solution by Hydrogenomonas. Ni uptake by the Ni-requiring H_2 autotrophic bacteria was rapid (within 60 min), energy dependent (inhibited by cyanide, azide, and arsenite), and specific for Ni (Tabillion and Kaltwasser, 1977).

In microorganisms other than the H_2 autotrophs which show measurable Ni uptake, Ni is translocated by the same mechanism which translocates Co (Katayama, 1961; Webb, 1970a), or more generally that which translocates required divalent cations (Mg, Mn, Co, and Zn) (Furhmann and Rothstein, 1968a; Webb, 1970b). Such uptake systems are stimulated by PO_4, but interpretation of the effect as due to polyphosphates as the

absorptive sites (Skaar, Rystad, and Jensen, 1974), or to the involvement of oxidative phosphorylation in translocation (Webb, 1970a) has differed. In these systems Ni uptake was rapid (saturated within 10 min) and energy dependent. These reported Ni translocation systems were thus not involved in Ni uptake by A. marinus in UB-M9 (Table 37). Substantial accumulation of Zn by A. marinus has been demonstrated (Jones, Royle, and Murray, 1976). It was thus unlikely that Ni and Zn were translocated by a single system in this organism. A. marinus apparently has a Mg and Zn translocation system which can discriminate Ni. Indeed, the presence of enhanced Ni accumulation by killed or metabolically inhibited cells indicated an active Ni exclusion system. This separation of essential (Mg, Zn) from non-essential or toxic (Ni) metal uptake may explain the Ni resistance responsible for the original isolation of A. marinus.

The lack of Ni uptake, especially at higher Ni concentrations (Table 37) at which toxicity was manifested, had implications for the mode of toxicity of Ni to A. marinus. The lack of penetration of the cells indicated that the site of Ni action was at the cell surface (Ross, 1975). Previous physiological studies of A. marinus had also concluded Ni toxicity to be a cell surface effect (Gonye, 1972).

The uptake of metals by microbial cells, which are at the bottom of most aquatic food webs, can act as the first step in the biomagnification of toxic materials. In view of the low magnitude of uptake of Ni by A. marinus, even in

non-chelating media, it was unlikely that A. marinus could act to biomagnify Ni in the environment.

The validity of the concept of concentration factor (CF) (Bowen, 1966) was questioned. As usually reported, CF is the concentration of metal in dry biomass divided by the concentration in the medium. It reflects the extent to which the organism has concentrated the metal from its environment. However, a report on Co uptake by E. coli in which CF was computed on the basis of wet cell mass (Katayama, 1960) led me to question the dry cell mass based CF. If a cell neither concentrated nor rejected a metal, its concentration in the cell would be identical to the medium. However, on drying, the metal within the cell would be retained and the net concentration increased by a factor equivalent to the cell water content. A CF based on dry cell mass for a cell of 90 % water content would be 10 for a cell which in fact did not concentrate the metal.

I concluded that the usually reported CF is invalid, and overstates the extent to which organisms concentrate substances from their environments by a factor of 5 fold or more. A valid CF can be calculated only when the organism's water content is accurately determined. This was impossible with A. marinus but a calculated water content allowed calculation of a corrected CF (Table 37). At the highest Ni concentrations tested the corrected CF approached 1.0 indicating that the concentration of Ni within the cells was equal to that outside. As the gram-negative cell wall contains a variety of potentially metal binding components external to

the cytoplasmic membrane, at which active metal exclusion would occur, the cell could actively exclude Ni without the whole cell CF being less than 1.0.

As was noted in the Results on TRIS- and UB-M9 media, there were striking differences between the Ni toxicity response of A. marinus in defined medium as compared to complex (2216E LN) medium. The differences are summarized in Table 42. In 2216E LN there was a typical bacterial toxicant response (Lamanna and Mallette, 1965; Bowen, 1966). In such a response, not limited to metals (Brock, 1970), at concentrations above a toxicity threshold both the growth rate and yield decreased over a fiarly narrow range of concentrations (typically one decade for metals) to a dose above which no growth occurred. Additionally, in 2216E LN, the lag time of the culture did not change, even at nearly totally inhibitory Ni doses. Reports of similar metal toxicity responses were found for bacteria (Sadler and Trudinger, 1966; Schade, 1949; Burke and McVeigh, 1969), fungi (Golubovich and Rabotnova, 1974) and algae (Jensen, Rystad, and Melsom, 1974; Steeman-Nielsen and Wium-Andersen, 1971; Wium-Andersen, 1974; Erickson, 1972; Hannan and Patouillet, 1972; Braek and Jensen, 1976; Sunda and Guillard, 1976).

Such a response is an a priori expectation of many who have studied microorganism metal toxicity, leading to the construction of metal toxicity assay by end-point growth yield determination, making the assumption that a reduced growth yield after a fixed period of incubation was equivalent to a depressed growth rate (Starr and Jones, 1957; Webb, 1970a; Zimmerman, 1971; Rosko and Rachlin, 1975; Arcuri and Erlich, 1977).

Table 42. Effect of toxic Ni concentration on A. marinus grown in complex and defined media.

	2216E LN[1]	UB-M9[2]
Growth yield	strongly depressed	little or no depression
Growth rate	strongly depressed	moderately depressed
Lag time	no change, inoculum dependent	strongly extended, inoculum dependent
Abnormal morphology	megalomorphs	slight size increase grainy cytoplasm

[1] Figures 11, 12, 13

[2] Tables 17, 19

The pattern of growth response to Ni of A. marinus in UB-M9 presented a distinct difference from this toxicant response. In defined medium Ni toxicity was expressed not as a decreased growth rate and yield but as an increased lag time (Table 19). Even at strongly inhibitory Ni concentrations the growth rate was depressed by a factor of four or less and the growth yield was essentially normal. By contrast, the lag time increased proportionately with the Ni concentration.

There was also a difference in the induction of abnormal morphology by Ni between the two media. The megalomorphic response expressed in 2216E LN was completely absent from UB-M9. In 2216E LN the maximum cell size was governed inversely by the size of the inoculum. Both that effect and the absence of megalomorphs in glucose-grown cells were proposed to arise from a single mechanism, that of a specific Ni-induced metabolic blockage. This model supposed that the immediate effect of Ni toxicity was to inhibit both the induction of cell division and one or more synthetic pathways (such as protein synthesis) which led to cell growth, without inhibiting other aspects of catabolism. Specific metabolic blockage by Ni is known in yeast, where ethanol dehydrogenase was inhibited by Ni concentrations which did not inhibit respiration (Fuhrmann and Rothstein, 1968b).

The inhibited A. marinus cell would not grow or divide. This was the case during the Ni-induced lag phase in UB-M9. If however, the medium provided preformed biosynthetic intermediates (such as amino acids or lipids) which bypassed the blocked pathway, cell growth could continue without cell division.

This was the case with 2216E LN. The spheroid megalomorphic cells would thus be produced if the cell was genetically predisposed to the formation of amorphous, rather than filamentous, megalomorphs (Long, Slayman, and Low, 1978). If the bypassing nutrients were in limited concentration in 2216E LN, a large inoculum would provide so many cells in competition for the nutrients that cell growth occurred for a limited period until exhaustion of the limiting nutrient, and the cells ceased growth at a small size. This was the case with the largest inocula used (Table 16). As inocula decreased the amount of nutrient on a per cell basis increased and the maximum cell size would increase. Inocula of 10.0, 1.0, and 1.1 ml EQ gave cell volumes of 1:5.4:27 (4, 7, 12 μm diameter) respectively. At inocula below 0.1 ml EQ, other factors, such as the inability of diffusion to move metabolites from synthesis to use points, acted to limit maximum cell size. The time course of megalomorph enlargement (Figs. 14 - 16) also supported this model. Grown on glucose, the bypassing preformed metabolite was unavailable, and cells showed little or no morphologic change (Fig. 31).

A second model for megalomorph effects, that of neutralization of Ni toxicity by the inoculum either by uptake or by cell products which reduced Ni toxicity, thus reducing the magnitude of the cell size change with larger inocula, was rejected. However, Ni uptake in 2216E LN did not occur. If a neutralization mechanism was acting, an effect of larger inocula would be to shift the Ni concentration giving maximum effect. But in fact,

maximum cell size of A. marinus occurred at 4 X 10^{-4}M Ni, independent of inoculum (Table 16).

The absence of a metal-dependent lag phase in 2216E LN may have been a result of megalomorph formation. Though megalomorphic cells were viable, and capable of reversion when placed onto Ni-free medium (Fig. 20), the commitment of cells to megalomorphy may have injured the cell such that it was incapable of ultimately derepressing division in the presence of the initially toxic Ni concentration.

Significant features of the Ni-induced lag phase of A. marinus are summarized in Table 43 in comparison to other reported metal-induced lag phases in microorganisms. Such reports are relatively rare in the literature, though it was unclear whether this was due to the effect being rare, due to failure of experimenters to extend experiments sufficiently to observe a present lag phase, or due to failure to report an observed lag phase. In some cases metal-dependent lag phases were evident from published data without having been commented on by the authors (Picket and Dean, 1976a). Other metal-induced lags include: Thiobacillus ferrooxidans due to several metals including Ni (Tuovinen, Niemela, and Gyllenberg, 1971; Barbic, 1977), P. fluorescens due to Be (MacCordick, Hornsperger, and Wurtz, 1975), Saccharomyces sp. due to Cu, Zn, and Cd (Bartlett, Rabe, and Funk, 1974).

In the presence of toxic concentrations of Ni in M9-type media, a lag phase was induced whose length was directly proportional to Ni concentration and inversely proportional to inoculum size. There was no evidence for cell adaptation

Table 43. Comparison of reported metal-induced lag phases with *A. marinus* Ni-induced lag.

	Cd - *E. coli*[1]	Hg - various microorganisms[2,3]	Cu - *Chlorella*[4]	Ni - *A. marinus*
Lag time proportional to metal concentration?		yes	yes	yes
Proportional to inoculum size?	no	inversely proportional	inversely proportional	inversely proportional
Cell adaptation?	yes	yes	?	no
Medium conditioning?	no	yes	yes	yes
Metal removal?	no	yes	no	no

1 Mitra *et al.*, 1975

2 Hayakawa, Kusaka, and Fukui, 1975

3 Ben-Basset and Mayer, 1975, 1977; Kamp-Nielsen, 1971

4 Steeman-Nielsen and Wium-Andersen, 1970; Steeman-Nielsen and Kamp-Nielsen, 1970

during lag, nor was the metal lost from solution by uptake or precipitation. Conditioning of the medium in the form of reduced Ni toxicity to a subsequent inoculum did occur during lag. The three other lag phase phenomena summarized in Table 43 bore both similarities to and differences from A. marinus - Ni lag.

The Cd-induced lag phase of E. coli differed from that of A. marinus in at least four of the properties listed. The phenomenon reported by Mitra et al. (1975) was induced by 3 X 10^{-6}M Cd in a defined medium. The lag phase was induced by Cd mediated single strand breaks of the cell DNA (Mitra and Bernstein, 1978), and represented the time necessary for cellular repair, and the induction of Cd resistance mechanisms such that Cd exposed cells gave no Cd-induced lag when reinoculated into Cd containing medium. Characteristic of the occurrence of cell adaptation in the termination of lag was the insensitivity of the lag time to variations of inoculum size.

Hg-induced lag phases, both in Pseudomonas sp. (Hayakawa, Kusaka, and Fukui, 1975) and in Chlorella and Chlamydomonas (Ben-Basset and Mayer, 1975, 1977; Kamp-Nielsen, 1971) was inoculum dependent, proportional to metal concentration, and involved cell adaptation, medium conditioning, and removal of metal from the medium. Inhibitory concentrations of Hg prevented cells from dividing, but they remained viable. During the ensuing lag phase an inducible Hg^{++} reducing system was activated, with the Hg^{o} formed lost from the medium by volatilization. When the Hg^{++} concentration of the medium had

fallen below a toxicity threshold, growth commenced with normal kinetics. As the medium Hg content was reduced during lag both metal loss and medium conditioning occurred. Further, as Hg-grown cells had the Hg volatilization system preinduced, such cells showed a reduced lag on reinoculation into Hg containing medium and thus were adapted. As more Hg or fewer cells meant more Hg to be volatilized per cell, the lag was proportionate to Hg concentration and inversely proportional to inoculum size.

The third case, that of Cu-induced lag of Chlorella pyrenoidosa, which was found in media free of chelating agents, was a metal concentration dependent lag followed by growth with normal kinetics. Cell adaptation effects were not reported. Medium in which the algae had grown showed reduced toxicity, an effect which was mimicked by EDTA.

The A. marinus-Ni lag phase was clearly different from the E. coli-Cd lag. The growth of A. marinus in UB-M9 showed no evidence for cellular adaptation to Ni (Tables 30, 31), unlike E. coli (Webb, 1970b) which was capable of substantial adaptation to Ni and Co. Further, the inverse inoculum dependency of the lag of A. marinus (Table 27) with its implication of cell to cell interaction was incompatable with intracellular adaptation (Mitra et al., 1975). These features argued for an extracellular mode of lag phase elimination. Ashida (1965) in his review of microbial metal resistance indicated two non-adaptive mechanisms: "destruction" of the metal, and the production of a metal binding agent. Destruction encompassed mechanisms of physical removal of the metal from the cell's

environment. Removal mechanisms include volatilization (i.e. of Hg^0), cellular uptake, and precipitation by H_2S (Ashida and Nakamura, 1954; Sadler and Trudinger, 1967). A. marinus did not produce H_2S (Table 6), or take up Ni. Although a volatile (and carcinogenic) form of Ni, Ni carbonyl, does exist (Anonymous, 1975; Sax, 1975), Ni was not lost from the medium during lag (Table 36), nor have I found any reference to Ni carbonyl production by bacteria.

It was thus most likely that the mode of recovery from lag phase by A. marinus was by the production of an extracellular metal binding agent. The A. marinus inoculum remained metabolically active, and thus able to produce a metal binding agent, during Ni-induced lag as shown by the development of the 215 nm UV absorbing peak (Figs. 47 - 49) and of the "metabolite peak" of the polarograms (Fig. 58), perhaps two manifestations of the same glucose metabolite. Direct evidence for extracellular medium conditioning was provided by the Ni toxicity bioassay test (Tables 23, 24) which showed reduction of the effective Ni concentration during lag.

The lag phase of bacteria in the absence of toxic agents has been examined by Shida, Komagata, and Mitsugi (1975). They defined a value, the L_{10}, as the reduction of lag time as the inoculum size was increased 10-fold. The L_{10} equalled the generation time. A regular L_{10} was definable for a number of bacteria including P. sp., E. coli, A. faecalis, and Proteus ovalis but not for B. subtilis or A. globiformis. Examination of the inoculum size - lag data for both 2216E LN

and UB-M9 media showed no regular magnitude of lag time reduction with inoculum size increase of A. marinus.

The bacterial lag phase usually is attributed to the necessity for an old inoculum or one from a different medium to readjust its metabolism, induce non-constitutive enzyme systems, and to synthesize biosynthetic machinery (Stanier, Doudoroff, and Adelberg, 1970). However, lag induced by toxic or unfavorable agents in the cell's environment may be a widespread phenomenon. Critical inoculum size, below which growth will not occur, is frequently encountered in the cultivation of fastidious microorganisms (Jannasch, 1965). The minimum viable inoculum was interpreted as evidence for the ability of the organisms to modify an initially unfavorable environment by the metabolic products of the cells to a condition suitable for growth. Below a minimum inoculum, insufficient cells could not effect the modification and growth did not occur. The growth of A. marinus in UB-M9 medium may have been governed by initially inhibitory conditions, perhaps due to the background metal content of the medium (Table 2). The usual interpretation of a lag phase predicts that an inoculum from an actively growing culture shows little or no lag in the same medium. Although the inoculum used for UB-M9 experiments was taken from an actively growing (late logarithmic phase) culture, there was an appreciable lag in UB-M9 medium without added Ni (Fig. 28). Sources of lag other than background metals included the shock of centrifugation and resuspension of the inoculum, and the reduced pH of the starter (c. pH 4 to 5) at the time of inoculum harvesting. Inhibition of bacteria by the background

concentration of metals may act *in situ* (Gillespie and Vaccaro, 1978). A background metal-induced toxicity may also have been responsible for the difficulty encountered in reproducibility of UB-M9 growth ranges (Table 20).

The derivation of the HMBO detection system from that of Stolzberg and Rosin (1977), and the basis of the selectivity of the technique for ligands of high stability (pK_m = c. 18) has already been discussed. A similar rational was used to partition the Cu, Pb, and Cd content of seawater into extractable and nonextractable fractions. I found QH_2O and KASW to be essentially zero in HMBO's. In both UB-M9 and 2216E LN media there was an appreciable apparent HMBO content (Fig. 56), but because there was no well defined saturation point in 2216E LN medium and no saturation whatever in UB-M9, it was impossible to quantitate a single HMBO molarity. In 2216E LN there was a saturation (c. 5×10^{-5}M Ni) but it was reached by a gradual leveling of the curve of Ni added versus HMBO detected. In UB-M9 medium no leveling of the curve occurred, allowing no single HMBO molarity to be defined. It was evident from these data that the technique of HMBO detection as proposed by Stolzberg and Rosin (1977) must be applied with caution, particularly when applied to undefined solutions of ligands of varying avidities, such as peptone based media, or natural samples. A molarity of strong metal binding organic matter defined by measurement at a single added metal concentration may yield an unreliable result.

Despite these apparent inadequacies, it was possible to use the technique to define relative changes of the apparent

HMBO molarity of UB-M9 medium during lag and growth (Fig. 57). For A. marinus grown in the presence or absence of Ni, there was an appreciable increase of the HMBO molarity of the medium during lag and growth. However, as the HMBO molarity, even in the control medium, never approached the molarity of Ni present, such strong metal binding ligands as were quantitated were not the sole mediators of the medium conditioning. The production of a small amount of strong ligands by A. marinus during its lag phase indicated that the bacterium produced at least two metal binding agents, one strong enough to be detected as an HMBO but insufficient to quantitatively account for Ni detoxification, and the other too weak to be detected as an HMBO but produced in sufficient quantity to account for Ni detoxification.

The successful use of dialysis and/or ultrafiltration to detect metal-organic complexation has been reported (Giesy and Paine, 1977; Gjessing, 1973; Giesy, 1976; Hood, 1967). A significant fraction of the soluble content of various transition metals in natural samples were in the form of complexes which would not pass through dialysis or ultrafiltration membranes. Giesy (1976) and Giesy and Paine (1977) partitioned the soluble metals into fractions of defined effective molecular weight by the use of ultrafilters of graded effective porosities. My negative results in dialysis and ultrafiltration experiments may have indicated a low effective molecular weight of the medium conditioning agent.

The gel filtration technique detected and isolated organometallic complexes in natural waters (Gjessing, 1973;

Mantoura and Riley, 1975; Means, Crerar and Amster, 1977). A weak organometallic complex might dissociate during gel filtration chromatography with the organic ligand eluting unnoticed with other organic compounds while the Ni adsorbed to the resin would elute separately in the salts peak. The absence of the appearance of low elution volume (i.e. organic complexed) ^{63}Ni during the lag phase and growth of A. marinus on ^{63}Ni labeled UB-M9 medium may have been due to one or both of two factors. Firstly, if the effective molecular weight of the complex formed was less than several hundred daltons, it would elute with the salt peak as it was too small to be separated. Secondly, if the complex was sufficiently weak, it would have dissociated on the column and thus not have been detected.

Polarographic techniques, particularly the differential pulse anodic stripping voltammetry and differential pulse polarography procedures, are highly sensitive to the concentration of the metal and to its speciation (Ernst, Allen, and Mancy, 1975). These techniques have been applied to the characterization of metal-organic complexation for several metals, notably Cu, in natural waters without intermediate concentration steps (Chau, Gachter, and Lum-Shue-Chan, 1974; Brezonik, Brauner, and Stumm, 1976). The technique has been extended to the calculation of apparent stability constants of natural ligands by Ernst, Allen, and Mancy (1975) and Shuman and Woodward (1977). Polarographic techniques have also been applied to the speciation of heavy metals, notably Cu, in microbial growth media (Avakyan and Rabotnova, 1966; Gachter, Lum-Shue-Chan, and Chau, 1973).

Organic complexation of a metal can have two effects on the polarographic response of a metal. Complexes such as those formed by high stability ligands, such as EDTA, are not reduced at the mercury electrode and are thus not measured (Brezonik, Brauner, and Stumm, 1976). My data show EDTA formed a 1:1 complex with Ni (Table 34) and the resulting equimolar amount of Ni disappeared from the polarograms (Fig. 29). Alternatively, a ligand may allow the metal to reduce at the electrode, but with the reduction potential changed because the metal must be removed from the complex prior to reduction, requiring higher energies. The reduction potential will be shifted to a greater negative potential (Brezonik, Brauner, and Stumm, 1976). This effect may have been responsible for the -1.26 v peak with histidine-Ni complexes (Fig. 41). During the lag phase of A. marinus grown in UB-M9 medium, both types of changes occurred in the polarograms of the medium (Figs. 66, 67). The height of the peak decreased and its reduction shifted to a more negative potential. Whether this was indicative of more than one ligand, or whether a single ligand could cause both effects was unclear. The magnitude of the peak size decrease was adequate to account for the proposed medium conditioning effect, with the Ni peak molarity falling to 4×10^{-6} and 2×10^{-5}M Ni for the 2×10^{-5} and 5×10^{-5}M Ni media, respectively, prior to the end of the A. marinus lag phase (Table 41).

The measured peak size did not fall to the same concentration for each culture, i.e. to the hypothetical threshold Ni concentration (equivalent to the minimum inhibitory dose,

5 X 10^{-6}M Ni for experiments in the same medium batch as the two above experiments) below which A. marinus growth and division was derepressed. The result was of concern. Several factors may have accounted for this effect. Firstly, it was impossible to monitor the cultures polarographically past a point, prior to the end of the lag phase, at which the metabolite peak encroached over the Ni peak. It was possible that the reduction of the peak size of the 5 X 10^{-5}M Ni cultures continued further than monitored in the experiment prior to growth induction. Secondly, if there was more than one complexing agent produced, one of which acted to reduce the peak height while the other weaker agent shifted the peak potential, the actual reduction in effective Ni concentration in the 5 X 10^{-5}M Ni cultures may have been more than indicated by the peak height reduction alone. Difference between the two Ni concentrations may be due to different relative amounts of the complexing agents produced at different Ni concentrations. Note that growth of A. marinus also caused Ni peak size decreases in 2216E LN medium (Fig. 24).

Metal-ligand stability constants can be determined from polarographic data. The Ligane equation requires that the number of electrons transferred in reduction of the metal, n (2 for Ni), the number of ligand molecules per metal ion, j, and the ligand concentration, $[L]$ be known (Ernst, Allen, and Mancy, 1975). $[L]$ can be calculated from the difference between the known Ni concentration of the medium (M_a) and the concentration measured polarographically (M_m), if the ligand number is known: $[L] = [M_a] - [M_m] \times j$. The Ligane equation

was solved for the stability constant as follows: $\log B_j = n/0.059\ (E_{1/2} - j \times 0.059/n \times \log [L])$. Since the complexing agents acting as ligand in the conditioning of UB-M9 were unknown, the value of j was estimated. The most probable values for j were ½, 1, and 2, corresponding to two metal ions per ligand (as proposed for histidine), 1:1 (as for EDTA), and two ligand molecules per metal ion, respectively. For those values of j, the corresponding $\log B_j$ values were: 3.28, 5.37, and 9.29, based on the 136 h data point of the 5×10^{-5}M Ni polarography experiment (Table 41): $E_{1/2} = 0.025$ v, $M_m = 2 \times 10^{-5}$M. Obviously, the calculated value of the stability constant is highly dependent on the value of j. The stability constant corresponding to a ligand number of 1 (5.37) was comparable to values for glycine (6.5) but lower than histidine (8.8) (Sillen and Martell, 1971). Reported stability constants for ligands in natural waters include Cu-fulvic acid, 8.68 (Manning and Ramamoorthy, 1973), Cu-undefined dissolved organic matter, 4.5 to 5.7 (Shuman and Woodward, 1977), Pb-humic acid, 14.8, Cu-humic acid, 16.8 (Ernst, Allen, and Mancy, 1975). The metal complexing agents detected polarographically in conditioned UB-M9 were thus of comparable stability to ligands of natural waters, and to artificial ligands which gave growth effects comparable to conditioned medium. On the basis of the calculated stability constants of the A. marinus metal complexing agent(s), it is most likely that the physical separation experiments failed because the complex was weak enough to dissociate during purification, and thus its molecular weight is unknown.

The polarographic pattern of 2216E LN medium may be understood in light of the later polarographic data accumulated, particularly the polarographic effect of histidine (Fig. 41), and of the effect on pulse polarography of organic compounds adsorbing to and coating the mercury drop electrode (Brezonik, Brauner, and Stumm, 1976). Adsorption effects due to compounds such as gelatin, surfactants, and starch included shifting of the peak to more positive values. The effects were studied with ASV at a hanging drop electrode; DPP at a dropping mercury electrode was less effected by absorption due to the constant renewal of the electrode surface at each drop. The polarographic pattern of Ni in 2216E LN (Fig. 24) may be explained in terms of both absorption of proteinaceous compounds from the medium onto the electrode causing shifting of the peak to more positive potentials and/or of the contribution of amino acids, such as histidine, which gave multiple peaks both above and below the free metal reduction peak.

The identity of the complexing agent(s) is unknown. The acidic metabolite produced by A. marinus from glucose, which was probably identical to the 215 nm UV components and to the polarographic metabolite peak, may have been identical to the complexing agent. Attempts to identify the glucose metabolite were unsuccessful. It was not gluconic acid, or a derivative of that acid (UV Spectroscopy, Pulse Polarography of UB-M9) which could arise from the early steps of the Entner-Doudoroff pathway by which A. marinus has been shown to catabolise glucose (Baumann and Baumann, 1973; Sawyer, Baumann, and Baumann, 1977). Nor was it pyruvic acid or any of the other

organic acids from the Krebs cycle which are produced by marine bacteria (Cho and Eagon, 1967; Ruby and Nealson, 1977) (detection attempted by gas chromatography; Hines, personal communication). Other products possible as medium conditioning agents include amino acids and small proteins.

Such bacterially produced metal complexing agents may have ecological significance. Some speculation based on the known role of metal complexing agents in the aqueous environment can be made, leading to the posing of the questions necessary to determine an ecological role for A. marinus metal complexing agents. Significant or major fractions of the heavy metal ions in natural solutions are present as organometallic complexes (Duinker and Kramer, 1977; Ramamoorthy and Kushner, 1975; Chau and Lum-Shue-Chan, 1974; Davey, Morgan, and Erickson, 1973; see also Literature Review). Size fractionation of natural ligands has shown important fractions with molecular weights of less than 500 daltons (Giesy, 1976). Organic metal complexing agents have a major role in the regulation of phytoplankton productivity (Barber and Ryther, 1969; Barber, 1972), and the triggering of blooms (Anderson and Morel, 1978). Metal complexation may function similarly for bacteria in natural waters (Jones, 1971; Milanovich, Wilson, and Yeh, 1975).

The ability of microorganisms to produce a variety of compounds capable of complexing metals is well demonstrated (Perlman, 1965). Bacteria, phytoplankton and zooplankton, and aquatic macrophytes release significant amounts of organic carbon in forms such as organic acids and amino acids (Webb

and Johannes, 1967; Fogg, 1952; Penhale and Smith, 1977; Williams and Yentsch, 1976; Sieburth and Jensen, 1970; Berman and Holm-Hansen, 1974). There are also reports indicating the production of specific chelating agents by phytoplankton (Khailov, 1964; Murphy, Lean, and Nalewajko, 1975; Simpson and Neilands, 1976). In addition to the Fe-transport siderophores, other bacterial products, such as cell surface components, may also be significant sources of metal binding agents (Corpe, 1975; Costerton, Geesy, and Cheng, 1978).

Thus, the metal binding agents produced by A. marinus may have an environmental role. To properly assess their function, several additional questions need be answered: Do other bacteria produce the same or similar metal complexing agents? Is the metal-complexing agent a product of normal metabolism or a specific response to Ni, and if so, is it produced in response to other metals? Are these complexing agents produced in sufficient quantity to have a role in the speciation of metals in natural waters or are they only of significance in environments which are diffusion limited, such as sedimentary microenvironments?

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APPENDIX I

EQUIPMENT SUPPLIERS

Manufacturer	Address
American Sterilizer Co., Inc. (AMSCO)	Erie, PA.
Amicon Corporation	Lexington, MA.
Applied Metals Research (AMR)	Bedford Lake, OH.
The Baker Co., Inc.	Sanford, ME.
Baltimore Biological Laboratories (BBL)	Cockersville, MD.
Beckman Instruments, Inc.	Fullerton, CA.
Better Built Machinery Corporation	Saddlebrook, N.J.
Bio-Rad	Richmond, CA.
BoLab, Inc.	Derry, N.H.
Cahn Instruments, Inc.	Cerritos, CA.
Calbiochem	La Jolla, CA.
Ciba	Summit, N.J.
Cole Parmer Instrument Co., Inc.	Chicago, IL.
Corning Glass Works	Corning, N.Y.
Difco Laboratories, Inc.	Detroit, MI.
Dow Chemical, Inc.	Indianapolis, IN.
Dupont	Wilmington, DE.
Eastman Kodak Co., Inc.	Rochester, N.Y.
EDAX	Prairie View, IL.
Fisher Scientific, Inc.	Fair Lawn, N.J.
Houston Instruments, Inc.	Austin, TX.
Instrumentation Laboratory	Lexington, MA.
J. T. Baker, Inc.	Phillipsburg, N.J.

APPENDIX I

Continued

Manufacturer	Address
Kimberly Clark	Neenah, WI.
Ladd Research Industries	Burlington, VT.
E. Leitz, Inc.	Rockleigh, N.J.
The London Co.	Cleveland, OH.
Markson Science, Inc.	Boston, MA.
Mettler Instrument Corporation	Princeton, N.J.
Millipore Corporation	Bedford, MA.
Monroe (Litton Business Systems, Inc.)	Orange, N.J.
National Laboratories, Inc.	Montvale, N.J.
New Brunswick Scientific, Inc.	New Brunswick, N.J.
New England Nuclear Corporation	Boston, MA.
Nuclepore Corporation	Pleasanton, CA.
Olympus Corporation of America	New Hyde Park, N.Y.
Packard Instrument Co.	Downers Grove, IL.
Pharmacia Fine Chemicals	Piscataway, N.J.
Phillips Electronic Instruments, Inc.	Mount Vernon, N.Y.
Pierce Chemical Co.	Rockford, IL.
Polaroid	Waltham, MA.
Princeton Applied Research (PAR)	Princeton, N.J.
Reichert (Wild Heerbruggs Instruments, Inc.)	Farmingdale, N.Y.
Revco, Inc.	West Columbia, S.C.
Scientific Products, Inc.	McGaw Park, IL.
Ivan Sorvall, Inc.	New Town, CN.

APPENDIX I

Continued

Manufacturer	Address
Spectrum Medical Industries	Los Angeles, CA.
Texas Instruments, Inc.	Dallas, TX.
VWR Scientific, Inc.	Boston, MA.
Whatman, Inc.	Clifton, N.J.
Worthington Biochemical, Inc.	Freehold, N.J.
Yellow Springs Instrument Co.	Yellow Springs, OH.
Carl Zeiss, Inc.	New York, N.Y.